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Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.)

K. Klimaszewska[1*], C. Noceda[2], G. Pelletier[1], P. Label[3], R. Rodriguez[2], M.A. Lelu-Walter[3]

[1]*Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 du P.E.P.S., P.O. Box 10380, Stn. Sainte-Foy, Quebec, Quebec, G1V 4C7, Canada*

[2]*Laboratorio Fisiología Vegetal, Departamento Biologia de Organismos y Sistemas, Univesidad de Oviedo, C/Catedrático Rodrigo Uría, s/n, 33071, Oviedo, Asturias, Spain*

[3] *I.N.R.A., UR 588 Unité Amélioration, Génétique et Physiologie Forestières, 2163 Avenue de la Pomme de pin CS 4001 Ardon, F- 45 075 Orléans Cedex 2, France*

[*]Author to whom correspondence should be addressed: email:

kklimaszewska@cfl.forestry.ca

Running title: Young and aged *Pinus pinaster* SE cultures

Abstract *Pinus pinaster* (Ait.) somatic embryogenesis (SE) has been developed during the last decade and its application in tree improvement programs is underway. Nevertheless, a few more or less important problems still exist that have an impact on the efficiency of specific SE stages. One phenomenon, which had been observed in embryogenic tissue (embryonal mass, EM) initiated from immature seed, has been the loss of the ability to produce mature somatic embryos after the tissue had been cultured for several months. In an attempt to get insight into the differences between young cultures of EM (3 mo old since the first subculture) of *P. pinaster* that produced mature somatic embryos and the same lines of significantly increased age (18 mo old, aged EM) that stopped producing mature somatic embryos, we analyzed in both types of material the levels of endogenous hormones, polyamines, the global DNA methylation and associated methylation patterns. Additionally, we included in the analysis 2nd EM induced from mature somatic embryos. The analysis showed that the two tested genotypes displayed inconsistent hormonal and polyamine profiles in EM cultures of a similar phenotype and that it might be difficult to attribute one specific profile to a specific culture phenotype amongst genotypes.

Experiments were also undertaken to determine if the global DNA methylation and / or the resulting methylation pattern could be manipulated by treatment of the cultures with a hypomethylating drug 5-azacytidine (5-azaC). An aged EM was exposed to different concentrations and durations of 5-azaC and its response in culture was established by fresh mass increases and somatic embryo maturation potential. All of the analyses are new in maritime pine and thus they provide the first data on the biochemistry of EM in this species related to embryogenic potential.

Keywords DNA methylation; endogenous hormones; maritime pine; polyamines; 2nd somatic embryogenesis; somatic embryo maturation potential

Introduction

Somatic embryogenesis (SE) in conifers is at the base of all biotechnology products that potentially might be developed, including transgenic trees. At present it is a powerful tool for clonal propagation of superior genotypes and is being scaled up for commercial application in a few conifer species (Nehra et al. 2005; Klimaszewska et al. 2007). Maritime pine (*Pinus pinaster* Ait.) is of economic importance in regions of Western Europe. In France for example, the planting area of maritime pine covers 1.4 million ha, making this species the most prevalent in reforestation and afforestation, and in Portugal it represents 30% of forest species. Due to its economic importance it has been also introduced into Australia, Chile and Argentina.

The research on SE in maritime pine dates back to 1995 when Bercetche and Pâques published the first report on this subject. Subsequent papers dealt with improvements of SE protocols to achieve higher frequencies of initiation, somatic embryo maturation and plant production (Lelu et al. 1999; Ramarosandratana et al. 2001 a, b; Miguel et al. 2004; Lelu-Walter et al. 2006). Although the SE process can be managed for high efficiency, there are still several problems that exist and require a better understanding of the underlying causes.

One phenomenon, which has been observed by researchers culturing embryogenic tissue, particularly of pines, has been the loss of ability to produce mature somatic embryos after the tissue had been cultured for over a year. It is inherent to SE of conifers that the cultured embryonal mass differs in embryogenic potential depending on the line (genotype), which is influenced by a parental cross (Lelu-Walter et al. 2006). However, the eventual loss of this potential by a line that initially was productive has been associated with a change in the morphology of early somatic embryos (such as short suspensors) and in the cellular composition/organization of the culture (Breton et al. 2005). The authors of the latter

publication characterized in detail the embryonal mass (EM) of *P. pinaster* over a 6-mo culture period, pointing out a few factors that might be important in maintaining the mature somatic embryo production capacity for a longer period of time. Presently, a means to mitigate this shortcoming is cryopreservation of embryogenic tissue soon after initiation to preserve its original characteristics and to thaw the tissue periodically to avoid failure in plant production.

In an attempt to understand the change in embryo maturation ability of a culture, other authors determined and detected differences in the type and levels of polyamines (PAs) in embryo producing and non-embryo producing cultures of radiata pine (Minocha et al. 1999). PAs act as development modulators and have been implicated in plant embryogenesis (Galston 1983; Uribe-Moraga et al. 2004 and references therein). Presently, PAs are considered to be a new group of plant growth regulators that, contrary to classical hormones, play a dual role, structural and regulatory, and this may explain their high cellular content. Other important molecules involved in cell division and differentiation as well as plant development are phytohormones albeit present at concentrations of orders of magnitude lower than PAs (Ljung et al. 2004). Jourdain et al. (1997) compared the levels of cytokinins, auxin, abscisic acid, and some of their conjugates in embryonal mass and in callus of hybrid larch both derived from the same seed explant and found significant differences. In conclusion, the embryonal mass possessing the ability to produce mature somatic embryos and plants had a significantly different hormonal profile than callus reflecting their different morphogenic characteristics.

However, none of the above studies included more than one factor to characterize the cultures in the same (single) study and none of them compared EM of the same genotype (line) and of different ages and embryogenic potential. Therefore, there has not been a

sufficiently exhaustive approach undertaken to determine if the phenomenon of a lost embryogenic potential might be linked to a specific biochemical change in the cultures.

It has been evidenced in the literature that cultured plant cells may undergo somaclonal variation, which may manifest itself at various levels such as phenotypic, cytological, biochemical and genetic/epigenetic (Kaeppler et al. 2000). In principal, somaclonal variation is defined primarily as epigenetic change that alters gene expression pattern without changes in the DNA sequence (Russo et al. 1996; Kaeppler et al. 2000). One chief contributor implicated in epigenetics is DNA methylation, which in higher plants may amount to as much as 30% of all cytosine residues methylated at both 5'-CG-3' and 5'-CNG-3' sites (Gruenbaum et al. 1981) and also at 5'-CNN-3' sites (Tariq and Paszkowski 2004). Among other functions, DNA methylation plays an important role in gene expression regulation (reviewed by Finnegan et al. 1998; Paszkowski and Whitham 2001) and as such may cause epigenetic change leading to suppressed embryogenic potential. For example, hypermethylation of DNA was found to be associated with a lack of organogenic potential in radiata pine micrografts collected from mature *versus* juvenile trees (Fraga et al. 2002a). Other evidence that gene expression was linked to embryogenic potential came from work by Charbit et al. (2004) who distinguished four of 28 cDNAs of *Hevea brasiliensis* (rubber tree) that were differentially expressed in embryogenic and regenerating cultures *versus* non-embryogenic cultures.

In an attempt to obtain insight into differences between young cultures (3 mo old) of EM of two *P. pinaster* genotypes that produced mature somatic embryos and the same lines of significantly increased age (18 mo old, aged cultures) that stopped producing mature somatic embryos, we analyzed in both types of material the levels of endogenous hormones, polyamines, DNA methylation and associated methylation patterns, which may vary in different cultures in spite of a similar global methylation (Guo et al. 2007). Moreover, to

determine if the DNA methylation could be altered, we exposed the aged EM cultures to different concentrations of a hypomethylating drug 5-azaC and tested the ability of the cultures for somatic embryo maturation immediately after the treatment.

For comparison, we induced secondary SE from a few mature somatic embryos of the same lines and included these cultures in the above analyses. Secondary SE has been implicated in restoring / enhancing the embryogenic potential of a given line (Lelu et al. 1994) and therefore, theoretically, we expected the tissue to be biologically similar to the young embryogenic culture. Thus far, this study represents the most exhaustive approach yet to determine if there are detectable biochemical changes in conifer EM after prolonged culture periods associated with the loss of somatic embryo maturation.

Materials and Methods

Culture of EM. ***Embryonal masses of* P. pinaster *(maritime pine) were initiated from immature zygotic embryos and cultured according to the published protocol by Lelu-Walter et al. (2006). The concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) in modified Litvay's medium (Litvay et al. 1985) (MLV) was 2.2 and 2.3 µM, respectively. Sucrose was added at 30 g l^{-1} and gellan gum (Phytagel™, Sigma) at 4 g l^{-1}. The tissue lines were subcultured onto fresh medium every 2 wk.***

Maturation of somatic embryos. Early somatic embryos were matured according to the protocol described by Lelu-Walter et al. (2006). Briefly, approximately 75 to 100 mg fresh mass (f.m.) EM was suspended in 4 ml liquid MLV growth regulator-free medium supplemented with 3% sucrose and 10 g l^{-1} charcoal and poured over a filter paper disc (Whatman #2, 7.5 cm diameter) in a Büchner funnel. The drained and finely dispersed EM was then cultured on a filter paper placed on maturation medium (MLV supplemented with 80 µM abscisic acid (ABA), 0.2 M sucrose and solidified with 10 g l^{-1} gellan gum) for 12 wk.

Initiation of secondary somatic embryogenesis (2nd SE). Mature somatic

embryos (12 wk old) and young somatic seedlings (without a shoot) of the following lines: NM10; NM12; NM18; NM21, and MM25 were cultured to test their ability for secondary embryogenesis (Table 1). The medium for induction of 2nd embryogenesis was MLV that included either 9.5 µM 2,4-D and 4.5 µM BA or 28.5 µM 2,4-D and 4.5 µM BA. Subsequently only the EM lines initiated on MLV with 9.5 µM 2,4-D and 4.5 µM BA were subcultured onto MLV with 2.2 µM 2,4-D and 2.3 µM BA for proliferation on the filter papers and collected for maturation or analysis (see below).

Materials for determining hormone, polyamine and global DNA methylation levels, and DNA methylation patterns. EM of two lines NM18 and MM25 were lyophilised or frozen at –80°C until needed for the analysis. Four types of material were collected: (1) EM that was retrieved from cryostorage, and cultured for 3 mo, (2) EM that was continuously cultured for 18 mo since initiation, (2a) the same as in (2) but cultured for 9 or 14 days on a medium with various concentrations (ranging from 5 to 40 µM) of a hypomethylating drug 5-azaC, and (3) the 2nd EM initiated from mature somatic embryos of the respective lines also cultured for 3 mo (Fig. 1). Our hypothesis was that the 2nd EM should have biochemical similarity to the primary cryopreserved lines since the former showed restored somatic embryo maturation ability (Table 2). For each type of analysis, the EMs were collected 10 d after subculture unless stated otherwise.

Extraction, high performance liquid chromatography (HPLC) and quantification of hormones. Indole-3 acetic acid (IAA), indole-3 aspartate (IAAsp), zeatin (Z), zeatin riboside (ZR), isopentenyladenine (IP), isopentenyladenosine (IPA), abscisic acid (ABA) and abscisic acid glucose ester (ABA-GE) were extracted and purified from 30 mg lyophilized or from 700 mg f.m. embryogenic tissue according to the published procedure (Jourdain et al. 1997). The concentrated hormones were fractionated on a reversed-phase HPLC column (Lichrospher 5 µm, ODS 100, RP-18, Merck, Germany) thermo regulated at 40ºC. Sixty fractions were collected per sample and then evaporated to dryness in a speed-vac concentrator (Savant, USA). IAA and ABA were methylated with 500 µl of an ethereal diazomethane solution, evaporated again to dryness and finally all fractions were suspended in 1.5 ml distilled water. To determine hormone recovery rates, 100 µl aliquots of fractions containing tritiated hormones were subjected to scintillation counting (Beckman LS1801, Beckman, USA). All the subsequent steps including quantification by enzyme-linked immunosorbent assay

(ELISA) were as outlined in Jourdain et al. (1997). The hormones were quantified in four replicates of each sample of EM.

Extraction, HPLC and quantification of PAs. PAs were extracted in triplicate from either 25 mg lyophilized or from 580 mg f.m. material in 5% v/v cold perchloric acid (PCA) on ice. The extracts were centrifuged at 27000 *g* for 20 min. The supernatants, containing the free PAs (non-covalently bound PAs) (S fraction), were stored at -20°C until dansylation and each of the pellets was re-suspended in 1 ml of 1 M NaOH and stored at -20°C.

The hydrolysis of PA conjugates, PA dansylation, and reverse-phase HPLC were carried out as outlined in Fraga et al. (2002a). Briefly, aliquots of the supernatants and the pellets were acid hydrolyzed to release the PAs from both PCA-soluble (SH fraction) and PCA-insoluble (PH fraction) conjugates. The PAs in these extracts were dansylated and then resolved and quantified by reverse-phase HPLC (Waters chromatography, Waters, Milford, MA). Putrescine (Put), spermidine (Spd), spermine (Spm), 1,3-diaminopropane (DAP) and 1,7-diaminoheptane (HTD) were used as standards (Sigma, St. Louis, MO).

Statistical analysis. Hormone and PA concentrations were transformed to their square root to normalize the residuals and stabilize their variance (Steel and Torrie 1980). ANOVA was then performed on the transformed concentration of each hormone and polyamine separately. When the genotype × culture type interaction was significant, the effect of age on the transformed concentration was assessed separately for each genotype. Tests were conducted at the $\alpha = 0.05$ level. Means were back-transformed for presentation in the tables, which also include upper and lower limits of the back-transformed means.

Treatment with 5-azaC. Aliquots of filter sterilized 5-azaC stock solution were added to a medium, which was subsequently used for experimentation within 16 to 18 h. Three hundred mg f.m. of NM18-2 EM were cultured on a filter paper placed on the surface of a medium. Growth of EM was measured as f.m. increases during the culture period. After 9 and

14 d, the Ems were collected for the measurements of DNA methylation. The EM cultures treated for 9 d were also used for somatic embryo maturation according to the protocol described above (*Maturation of somatic embryos*).

Extraction of DNA and high performance capillary electrophoresis (HPCE). Genomic DNA from 180 - 200 mg f.m. tissue samples was extracted using DNeasy plant mini kit (Qiagen, MD, USA) according to the manufacturer's instructions with minor modifications. The extracted DNA was precipitated at –80°C in a solution of sodium acetate (2 M) and two volumes of cold isopropanol or 95% ethanol. The DNA pellet was then dried at ambient temperature followed by dissolution in 15 µl of water. The separation and analysis of deoxy-nucleosides was performed according to Fraga et al. (2000, 2002b). Briefly, DNA-hydrolysis was performed overnight at 37°C to separate the nucleotides and followed by a 2 h enzymatic hydrolysis to release nucleosides from nucleotides. HPCE procedure was carried out using an uncoated fused-silica capillary (Waters Chromatography A.A., 600 mm x 0.075 I.D., effective length 540 mm) and a capillary electrophoresis system (Capillary Ion Analyzer, Waters Chromatography A.A.). The global DNA methylation levels were determined in three replicates of each sample and expressed as the percentage of 5-methyldeoxycytidine (5-mdC) of the total deoxycytidines.

Statistical analysis of DNA methylation data. The variance of methylation percentages in the material treated with 5-azaC was analyzed with the GLM procedure for SAS (SAS Institute, Cary, NC). The control was compared with 5-azaC treated tissue through a contrast. Tests were conducted at the $\alpha = 0.05$ level.

Extraction of DNA and methylation sensitive amplification polymorphism (MSAP). Genomic DNA from approximately 200 mg f.m. tissue samples was extracted, precipitated and dried as described above. Afterwards, 500 ng of genomic DNA was digested with 10 U of EcoRI plus 10 U of HpaII or with 10 U EcoRI and 10 U MspI both in a final volume of 23 µl

containing 2.5 µl NEB buffer U (New England Biolabs, Beverly, MA) and incubated for 3 h at 37°C. Incubating the mix at 65ºC for 20 min stopped the reaction.

The digested fragments were then ligated to the adapters by adding 12 µl of ligation mixture containing 1 µl (1 U) T4 DNA ligase, 2.1 µM EcoRI adapter and 7.1 µM MspI/HpaII adapter, and incubated at 24ºC for 3 h.

For preselective amplification, 5 µl of the ligated sample, 5 µM EcoRI plus one selective base primer (+A) and 5 µM MspI/HpaII primer with no selective base were used for PCR reaction in a total volume of 50 µl. The reaction parameters were as follows: 94ºC for 5 min; 53 cycles of 94ºC for 1 min, 53ºC for 1.5 min and 72ºC for 1.5 min; 72ºC for 10 min.

Selective amplification was carried out in a volume of 7 µl containing 0.375 µl of EcoRI+A plus 2 more selective base primers (50 µM), labeled with infrared dye IRDye™ 700 or IRDye™ 800 (MWG Biotech), and 0.375 µl of MspI/HpaII plus 2 selective base primers (50 µM). The selective base sequences in each primer pair were (EcoRI-MspI/HpaII): AAC-CT, AAG-AT, AAG-CA, AAG-CT, ACA-CA, ACA-CT, ACA-TA, ACA-TA, ACC-AT, ACC-CA, ACC-CT, ACG-CT, ACT-CT, AGC-AT, AGC-CA, AGC-CT, AGC-TA, AGG-AT, AGG-CA, AGG-CT, AGG-TA, ATG-TA. The PCR reaction parameters were as follows: 94ºC for 5 min; 94ºC for 30 s; 15 cycles of 94ºC for 30 s, 62ºC for 30 s and 72ºC for 1 min; 23 cycles of 94ºC for 30 s, 56ºC for 30 s and 72ºC for 1 min; 72ºC for 10 min.

0.2 µl of each of the two differently labelled amplicons were loaded in each well of the gel per sample. The MSAP products were separated by electrophoresis and visualized on a two-dye IR2 DNA analyzer system (model 4200, LI-COR Biosciences, Lincoln, NE, USA) according to Myburg et al. (2001). Run parameters were as in Pelgas et al. (2005). The digital MSAP gel images were scored using the SAGA^{GT} software Version 3.0 (LI-COR Biosciences).

When bands were present only for one restriction enzyme combination, EcoRI/HpaII or EcoRI/MspI, they were designated as H or M bands, respectively. When bands were present for both restriction enzyme combinations, they were designated as HM bands.

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Results and Discussion

Initiation of secondary somatic embryogenesis (2nd SE). Mature somatic embryos of lines NM10; NM12; NM18; NM21, and MM25 all produced secondary (2nd) embryonal mass (Table 1). Of the two media tested, the medium with a standard concentration of 2,4-D (9.5 µM) was more beneficial than the medium with a higher concentration (28.5 µM). The highest response was manifested by the line MM25 from which 18 somatic embryos of the 22 cultured ones produced 2nd embryonal mass. A few germinating somatic embryos were also cultured and produced 2nd SE (Table 1).

It must be pointed out that the mature somatic embryos of *P. pinaster* even after 12 to 14 wk of maturation may have live embryonal cells attached to their radicles. These cells sometimes proliferated on the initiation medium and appeared as the 2nd initiation; however this tissue could be easily and entirely detached from the explant. In contrast, when 2nd SE initiation took place, the tissue could not be entirely

detached from the explant and usually after removing most of it a site of initiation was clearly visible on the explant. It is therefore imperative to closely examine every explant with proliferating EM to determine its origin and avoid a false positive result. Once the 2nd EM was initiated and proliferated, it was subcultured and maintained for 3 mo prior to the maturation experiments and analysis.

Somatic embryo maturation in young (1), aged (2) and 2nd EM (3) cultures. In genotypes NM18 and MM25, all of the above culture types were tested, whereas for NM10 and NM12 only the aged and 2nd EMs were available (Table 2).

The results were consistent across all the lines tested in that the maturation of somatic embryos occurred at a higher yield in the young EM cultures (Fig. 2A) and in 2nd EM cultures, whereas the aged cultures either did not produce any somatic embryos (Fig. 2B) or if they did, the yield was much lower (Table 2). The only exception was demonstrated by genotype MM25, which had similar embryo maturation ability, expressed as the number of well developed somatic embryos, from both MM25-2 and MM25-3 cultures.

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Hormones. ***Among all the hormones tested in both genotypes and materials, ABA was found to be at the highest mean titer compared with the mean levels of IAA, and the cytokinins (Z and IP) (Table 3). In general, compared with NM18, MM25 had lower overall levels of IAA (p = 0.02) and ABA-GE (p = 0.01) but the levels of cytokinins and their conjugates and ABA did not differ. Between genotypes, the hormone profiles in the three types of cultures did not show a consistent pattern. In MM25, all the hormones and their metabolites were at similar levels in young and aged cultures as well as in 2nd EM. Conversely, in NM18 IAAsp concentration was significantly higher in young culture (p = 0.004) compared with aged and 2nd EM. On the other hand ZR was at the lowest titer in young and 2nd EM compared with the aged cultures.***

In carrot cell culture a high titer of endogenous IAA was associated with a high embryogenic capacity (Sasaki et al. 1994; Jiménez and Bangerth 2001). However, another report said that this association was dependent on the stage and progression of SE and that the levels of IAA

decreased once SE was established (Michalczuk et al. 1992). In our study the elevated level of IAAsp in NM18-1 culture did not correspond to the level of IAAsp in MM25-1 in spite of the fact that both genotypes produced relatively high numbers of mature somatic embryos in young cultures (Table 2). Therefore it is not clear from this result if IAAsp must be at a certain threshold level for embryogenic potential manifestation and if so, why this was not the case for MM25. Possibly this might be indicative of the dynamic nature of IAA biosynthesis and metabolism in these two genotypes and / or this might be reflective of a difference in the progress of EM differentiation.

Overall, the hormonal profile in maritime pine was different from the published results on hybrid larch where the EM (capable of producing mature somatic embryos) was tested at three dates during a 2-wk proliferation cycle (Jourdain et al. 1997). High IAAsp, ABA-GE and IPA levels characterized the growing EM of the latter, whereas in maritime pine it was ABA and IAA (the latter in NM18 only) that were predominant. This difference in the endogenous hormone profiles between the two conifers would indicate that the embryogenic state might not be characterized by a uniform hormonal profile across the species and perhaps also genotypes. Furthermore, in hybrid larch varying concentrations of endogenous hormones were detected in the same material but collected at various dates during the 2-wk tissue culture cycle (Jourdain et al. 1997). On the other hand, in hybrid walnut (*Juglans nigra* x

regia) it has been shown that high IAAsp titer was a marker of embryogenic potential (Lucas 1996).

Based on the present and published results it seems that endogenous hormone profiling in maritime pine EMs that display different embryogenic potential is not straightforward and in addition to applying the most sensitive detection methodology (due to the low amounts of hormones in complex plant extracts and their different chemical properties, Ljung et al. 2004) it may also require temporal analysis of the cultured material to understand their role in SE.

PAs. ***The tissue samples of the two* P. pinaster *genotypes (NM18 and MM25) did not contain any detectable level of the Spd and Spm degradation product DAP in any of the tested material. The lack of this catabolic product was also noted in embryogenic and non-embryogenic cultures of* P. nigra *(C. Noceda, unpublished results), which might be indicative of the fast PA metabolism in pine cell cultures.***

The profiles of specific PAs were not the same in the two genotypes tested (Table 4). With respect to Put, the concentrations in all three fractions (S, SH and PH) did not differ significantly among any of the tested cultures within each genotype. In contrast, Spd and Spm profiles did differ in NM18 among the three types of cultures but not in MM25. In NM18, there were significantly lower levels of free Spd and Spm in young EM (1) compared with aged (2) and 2nd EMs (3). In SH fraction however, Spd titers did not differ but Spm was the lowest in aged EM. Interestingly, the 2nd EM did not have the same profile as the young EM culture as initially hypothesized. No other content differences were significant among the

cultures. Overall, NM18 cultures had higher total PA levels than MM25 cultures (1850 and 1648 nmol g^{-1} f.m. compared with 532 and 904 nmol g^{-1} f.m. in young and aged cultures, respectively), which was due to much lower titers of Spd and Spm in the latter.

PAs are essential in cell growth and division and free Put has been found the most abundant in proliferating EM of Scots pine and in Norway and red spruces followed by Spd and Spm (Sarjala et al. 1997; Minocha et al. 1993). However, towards the end of the exponential growth cycle as the increase in the tissue f.m. was arrested, the Put levels significantly decreased. Similarly, it has been found that free Put also played an important role in peach callus growth (Liu and Moriguchi 2007). The levels of Put basically paralleled the growth curve of the callus and reached its maximum at day 12 after subculture, which corresponded to the end of the exponential growth phase of callus. On the other hand, Spd and Spm titers reached maximal values earlier, during the lag phase of the callus growth.

In maritime pine the PAs were measured at only one time point during the culture cycle i.e., 10 d after subculture, therefore the dynamics of single PA changes were not followed because this study was focused on differences (or similarities) among EM cultures of different ages at any given time point during the growth cycle. The premise was that any difference, if detected, would persist regardless of the time interval after subculture.

Examples reported for other conifers suggested that the decrease in Put concomitant with an increase or a decrease in Spd was associated with a more differentiated state of the cultures. For example, in radiata pine during early stages of somatic embryo maturation, an increase in Spd relative to Put content occurred (Minocha et al. 1999). Conifer embryogenic cultures are highly heterogeneous and it has been shown that their cell type composition, presence of early somatic embryos and hence degree of organization may also vary with duration of the culture (Breton et al. 2005).

DNA methylation. DNA methylation analysis in plants has been approached by measuring either global levels of methylated cytosines (e.g., using HPLC or HPCE) or by examining specific DNA sites (e.g., MSAP) (Cervera et al. 2002 and references therein). In the present study we followed both approaches.

Global DNA methylation in young and aged EM cultures. Comparison of methylated cytosines in the EM cultures of different ages, in both genotypes, showed values between 17.8 to 19.1% (s.e. 0.37) and a lack of significant difference (Table 5). This is in contrast to a study on carrot where the methylation levels of three cell suspension lines ranged from 14.5 to 22.6% (Palmgren et al. 1991). The latter study also demonstrated that different cell types (vacuolar, meristematic, others) purified from the cultures had different methylation levels (from 22.2%, s.d. 0.2 to 25.4% s.d. 0.7); however no statistical analysis of the data was provided to support this conclusion. In another study involving carrot cell suspension, the ratio of methylated cytosines increased from 16 to 40 and 45% by increasing the concentration of 2,4-D in the culture medium from 0.5 to 2.0 and 5.0 mg l^{-1}, respectively, indicating its strong hypermethylating effect (LoSchiavo et al. 1989).

Global DNA methylation in NM18 cultures treated with 5-azaC. To determine if maritime pine aged EM cultures would be amenable to treatments of a DNA hypomethylating drug, the culture medium was supplemented with 5-azaC and its influence on NM18-2a EM growth after 9 d of culture and subsequently on the somatic embryo maturation ability was determined (Table 6, Experiment 1). The mean f.m. increase was greatly reduced by 5-azaC in the medium at concentrations greater than 5 µM. However, maturation of somatic embryos seemed to be slightly elevated from EM treated with 10 and 15 µM.

The DNA hypomethylating drug 5-azaC is a cytidine-analogue, in which the carbon at position 5 of the pyrimidine ring is replaced by nitrogen. This distinctive feature is responsible for inhibiting DNA methyltransferase (DNMT) by covalent binding to the DNA

molecules thereby making them unavailable for methylation. The result is demethylation of DNA after repeated replication, however depending on the drug concentration, eventual inhibition of DNA synthesis may occur. Another mechanism of 5-azaC action is incorporation into RNA, which causes ribosomal disassembly and consequently inhibits protein production. However, 5-azaC exhibits greater cytotoxicity during S-phase, supporting the greater importance of its effect on DNA (reviewed by Goffin and Eisenhauer 2002). The 5-azaC concentration dependent growth reduction that was observed in maritime pine cultures might be related to the drug's cytotoxic effect. Similarly, leaf disc explants of *Petunia hybrida* exposed to 5-azaC at 5 and 10 µM for 14 d had significantly reduced f.m. gain (by 60 and 80%, respectively) and shoot bud induction when compared with the untreated controls (Prakash and Kumar 1997). Likewise, in carrot cell suspensions, formation of normal embryos was inhibited by 90% when 1 µg l^{-1} of 5-azaC was incorporated into the culture medium (LoSchiavo et al. 1989). Together, these results indicated that a balance must be reached between the cytotoxic effect of 5-azaC and its hypomethylating ability, both of which affect mainly dividing cells.

Interestingly, in maritime pine, neither the 9 nor 14 d culture of NM18-2 on medium with 5-azaC influenced the global methylation level (Table 6). After a 9 d culture the tissue methylation was from 18.7 to 21.3% compared with the control at 18.0% and none of these values significantly differed ($p = 0.34$). Similarly, after 14 d treatment the methylation values were from 16.4 to 18.0% compared with control at 17.8%, which were also not different ($p = 0.49$). However, lack of quantitative differences in global cytosine methylation does not necessarily mean lack of qualitative modifications in the specific sites of DNA, which can be detected by studying the methylation pattern in different types of cultures and tissues (Finnegan et al. 1998; Baurens et al. 2004).

Methylation sensitive amplification polymorphism (MSAP). The first description of the MSAP technique to evaluate DNA methylation changes in plants regenerated *in vivo* was published for micropropagated banana by Peraza-Echeverria et al. (2001). Since MSAP is based on AFLP technology there is no requirement for prior genome information except for approximate genome size. Generally, this technique is recognized as suitable to evaluate epigenetic changes with respect to the methylation pattern, which may be associated with somaclonal variation. Our premise for examining this was that the same or similar global methylation levels may display different MSAP methylation patterns.

Most of the deliberations on the DNA methylation pattern changes described here were based on a report published by Xu et al. (2004). The principal idea was that the cells of the embryogenic cultures used in our study were heterogeneous with respect to the DNA-methylation patterns, especially the ones treated with 5-azaC, which is known to induce hypomethylation changes in the cell genome and is toxic to susceptible cells. The intensity of the same size bands may vary depending on the frequency of a specific DNA-methylation pattern in the analyzed group of cells. Consequently, only the very distinct banding patterns, which most likely existed in a sizeable group of cells, were computed. Furthermore, several distributions of methyl groups in 5'-CCGG-3', which correspond to the MspI / HpaII target sequences, might have existed but the MSAP detection limits allowed us to obtain information only about a few of them. For simplification, changes in the DNA-methylation that are deemed less likely to occur, such as the ones requiring more than one methylation change with respect to the control, were not included in the computing of the different types of DNA-methylation changes. Other considerations in interpretation of the banding patterns were as follows:

1) The substitution of HM band by M band always involves net methylation; in the case of methylation pattern homogeneity for the considered HM band in the cells of the

sample, the methylation occurs in inner cytosine (C) of 5'-CCGG-3' target sequences, with the outer C being non-methylated (neither methylation nor hemimethylation). The opposite implies the reverse methylation change. According to this criterion, we considered the banding changes HM → M as CC → CmC (downstream).

2) The disappearance of a band most likely implies the formation of a longer fragment, which is less likely to be amplified. Consequently, net disappearance of HM or M bands most likely involves outer C methylation, with the inner C being non-methylated (neither methylation nor hemimethylation) or already methylated (symmetric methylation), respectively. The opposite cases imply the reverse changes in the methylation status. According to these criteria, we estimated the outer C methylation events CC → mCC and CmC → mCmC.

3) The substitution of HM band in a control by H band in a sample implies hemimethylation of outer C. The opposite case involves the reverse DNA-methylation change. According to this criterion, we estimated the events of CC → hmCC.

Another limitation of the analysis was that the lack of bands for one population of cells could coincide with H, M and / or HM bands in another population of cells. The presence of HM band in both the control and the compared sample most likely implied no de-methylation (no change) or net methylation (HM change to M and to H in different cells, which could be combined also with HM or/and M disappearance in another population of cells) in the MspI / HpaII target sequences with respect to the control; nevertheless, because distinction between these different types of events was not possible, these cases were always quantified as "no change". Hence, underestimation of different types of net methylation/hemimethylation (or overestimation of de-methylation) events, especially in the tissues treated with 5-azaC, was unavoidable.

To estimate the proportion of the listed changes in 5'-CCGG-3' sequences, we expressed them as percent ratios of the number of specific cytosine single methylation events divided by the total number of CC plus CmC quantified in the corresponding control.

MSAP in NM18-1, NM18-2 and NM-18-3 cultures. The ageing of EM (NM18-1 *versus* NM18-2) was associated with epigenetic DNA changes involving net demethylation, restricted to the analysed types of methylation alterations. Demethylation of mCC was the most frequent event in the ageing cultures, but also methylation of CmC and inner C of CC (Fig. 3A, C). It seems that the net de-methylation of mCC is concomitant with the methylation of the closest to 3'non-methylated C of another 5´-CCGG-3´ target sequence. These C preferences for methylation would indicate that full methylation first requires methylation of inner C, in spite of the fact that a few cases of hemimethylation of outer C in CC sequences were also detected in aged cultures.

The same types of events as in NM18-2 were detected in NM18-3. Therefore, it would appear that both ageing and differentiation were qualitatively associated with the same type of methylation events. Alternatively, since the NM18-3 culture was derived from NM18-2 (through 2nd embryogenesis) it is plausible that the methylation pattern of NM18-2 was inherited by NM18-3.

In rose cultures de-methylation of mCC was the most frequent among quantified events when undifferentiated callus cells differentiated into embryogenic cells (Xu et al. 2004). The authors concluded that the modified cytosines might be critical for the somatic cells to acquire embryogenic ability, and the former were then inherited by the subsequent regenerants. Chakrabarty et al. (2003) detected changes in methylation pattern between embryogenic and non-embryogenic calli of Siberian ginseng using MSAP and several primer pairs. The authors postulated that in order for the embryogenic program to be expressed in callus cells, most likely another set of genes must be activated. Obviously, conclusions from

the above studies cannot be directly discussed in the context of the present study because the materials and the SE system in conifers substantially differ from those of angiosperm plants.

MSAP in NM18-2a cultures treated with 5-azaC. Net DNA-methylation, restricted to the analysed types of methylation modifications, was detected in the 5-azaC treated samples after 9 d of culture. The most frequent of the four quantified types of band changes in these samples was disappearance of M bands and/or disappearance of MH bands (which corresponds to MH or combination of M and H in different groups of cells, Fig. 3B) involving, without a doubt, net outer C methylation of 5´-CCGG-3´ target sequences. It seems that the detected net methylation in these samples was more prevalent in CC compared with CmC sequences (Fig. 3D). This latter alteration was detected at a low frequency only at 5-azaC concentrations higher than 5 µM in 9 d treatment or at the lowest drug concentration in 14 d treatment, in which net methylation was also computed with MSAP. These results might indicate that, at low drug concentrations or short exposures, groups of surviving cells regulated their genome function by more 5´-CCGG-3´ methylated sequences. The surviving cells and their precursor cells at the beginning of the culture were less susceptible to the DNA-replication interfering mechanisms of the drug, i.e., the cytotoxic blocking of DNA synthesis and probably the DNA-hypomethylation. It is plausible that the surviving cells in treated cultures might have been mainly non dividing cells, at least at the beginning of the culture, when the concentration of the applied drug was not significantly altered.

At longer exposure to the de-methylating drug (14 d) at the highest concentration (20 µM), net outer C methylation of CC (observed always at 9 d) was concomitant with net de-methylation corresponding to the following type of events: outer C de-methylation of mCmC and de-methylation of CmC (both changes imply a trend to full de-methylation of fully methylated target sequences -mCmC-). If such methylation and de-methylation changes occurred in the sufficiently numerous group of cells, the detection of each of these opposite

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trends could, to a great extent, correspond to one type of surviving cells in the same sample: methylation events in non dividing cells (less susceptible to the hypomethylating effect of the drug) and de-methylation events in late-proliferating cells.

The highest detected net methylation after 9 d and net hypomethylation after 14 d occurred at similar concentrations of 5-azaC (Fig. 3 B, D). This could mean that a certain correlation existed between early cytotoxic and later hypomethylating effects of 5-azaC. It must be pointed out that 5-azaC is highly unstable in neutral aqueous solution and has a short half-life (Goffin and Eisenhauer 2002). Thus, the degradation in the medium was unavoidable. Furthermore, this drug can be inactivated by deamination with cytidine deaminase (Lubbert 2000). It is therefore conceivable that both degradation and incorporation of 5-azaC into DNA influenced the amount of the drug in the medium over treatment time. At any given time, dividing cells were either dying or they were undergoing methylation changes depending on the residual 5-azaC concentration in the medium and on the proportion of cells entering the mitotic cycle. Theoretically, at the higher initial concentrations of the drug, the remaining amount after 14 d of culture might cause hypomethylation while the cytotoxic effect was minimized.

These results indicated that notwithstanding the lack of statistically significant differences in the global DNA methylation among the tested samples, there were qualitative alterations in specific target sequences as detected by MSAP.

In conclusion, we have demonstrated in this study that in *P. pinaster* it is possible to induce 2nd SE from mature somatic embryos and that these 2nd EM cultures, in the majority of tested genotypes, produced a higher number of mature somatic embryos compared with the aged cultures of the same genotypes. This approach to restore plant production ability from a culture that lost it may be useful if a tissue line has not been cryopreserved and / or if the

cryopreserved tissue was contaminated and / or if the remaining stored tissue stock is small and cannot be compromised.

The biochemical characterization of the three EM culture types (young, aged and 2nd) revealed different results for each of the two tested genotypes reflecting high variability in EM cultures in spite of similar phenotypes. Endogenous hormones did not have consistent profiles. Among MM25 cultures there were no significant differences in the mean hormone contents. However, in NM18, the young cultures were characterized by elevated IAAsp and lower ZR concentration compared with aged cultures. PA profiles were also inconsistent. In MM25 no significant differences in specific PAs were found among the cultures. However, in NM18, the aged EM had higher Spd and Spm levels and no difference in Put concentration. It is possible that different SE potential and different PA profiles together with ageing and 2nd SE process in NM18 and MM25 could be related to different profiles of IAAsp and ZR in these two genotypes.

Global DNA methylation levels were similar in all tested samples and ranged form 17.8 to 19.1% (s.e. 0.37). Nevertheless, the ageing of EM was associated with net DNA demethylation in 5´-CCGG-3´ target sequences as detected by MSAP. Demethylation of mCC was the most frequent event in the ageing EM, but also methylation of CmC and inner C of CC.

The treatment of NM18-2 with 5-azaC affected the type of methylation alterations in the target sequences depending on the drug concentration and exposure duration. The largest alterations in the DNA methylation detected by MSAP occurred after treatment with 15 µm 5-azaC; when the culture was exposed for 9 d, maximal hypermethylation was found, however upon 14 d treatment the drug caused maximal hypomethylation. The consequences of these changes on the subsequent culture behaviour remain to be established. In the above experiments we found a big variation in EM growth after culture on a medium with 5-azaC

and since this drug is known for its high cytotoxicity, other hypomethylating as well as hypermethylating drugs should be tested to determine their influence on the viability and embryo maturation potential of the treated, aged EM cultures.

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Table 1. Somatic embryogenesis initiation (number of responding / number of cultured explants) in *Pinus pinaster* mature somatic embryos and germinated somatic embryos after 10 wk of culture on media with two concentrations of 2,4-D, (A) 9.5 µM and (B) 28.5 µM both in combination with 2.3 µM BA.

Line		Medium A	B
NM10	se	10 / 28	0 / 27
NM12	se	3 / 22	0 / 7
NM18	se	4 / 14	0 / 31
	ge	2 / 8	0 / 5
NM21	se	18 / 60	1 / 68
	ge	1 / 13	1 / 12
MM25	se	18 / 22	Not tested

se – somatic embryo

ge – germinating embryo

Table 2. Somatic embryo maturation in original*, young (1), aged (2) and 2nd (3) EM cultures of *Pinus pinaster* (four genotypes).

Line and culture	Number of Petri dishes	Number of mature somatic embryos g^{-1} f.m.
NM10*	10	284
NM10-2	5	20
NM10-2	3	0
NM10-3A	5	250
NM10-3B	3	200
NM12*	8	72
NM12-2	3	0
NM12-3	3	100
NM18*	8	166
NM18-1	6	175
NM18-2	5	0
NM18-2	3	0
NM18-3A	5	300
NM18-3A	3	350
NM18-3C	5	>500
NM18-3C	3	350
MM25-1	3	460
	3	200
MM25-2	3	0
	3	30
	6	23
MM25-3	6	23

* Original lines were proliferated for 6 weeks.

Table 3. Titers of hormones (pmol g^{-1} f.m.) in EM of *P. pinaster* per genotype and culture type: young (1), aged (2) and secondary embryogenic (3). Numbers are back-transformed means and their 95% lower, upper confidence limits (CL).

		NM18			MM25		
			95% CL			*95% CL*	
Hormone	Culture type	Mean	*Lower*	*Upper*	Mean	*Lower*	*Upper*
IAA	1	215	*126*	*327*	27	*3*	*74*
	2	123	58	*211*	64	*21*	*131*
	3	57	*17*	*121*	26	*3*	*73*
	mean	**123**	*83*	*171*	**37**	*17*	*65*
IAAsp	1	78[a]	*48*	*116*	5	*0*	*18*
	2	31[b]	*13*	*56*	19	*6*	*40*
	3	20[b]	*7*	*41*	22	*8*	*44*
	mean	**40**	*27*	*55*	**14**	*7*	*24*
Z	1	14	*2*	*36*	23	*6*	*52*
	2	41	*16*	*76*	13	*2*	*35*
	3	15	*2*	*39*	23	*6*	*50*
	mean	**22**	*11*	*36*	**19**	*9*	*33*
ZR	1	4[a]	*1*	*7*	3	*1*	*6*
	2	21[b]	*15*	*29*	5	*2*	*9*
	3	5[a]	*2*	*9*	5	*2*	*8*
	mean	**8**	*6*	*11*	**4**	*3*	*6*
IP	1	26	*4*	*68*	22	*3*	*61*
	2	68	*27*	*129*	28	*5*	*71*
	3	23	*3*	*63*	58	*21*	*115*
	mean	**37**	*18*	*62*	**35**	*17*	*59*
IPA	1	4	*0*	*12*	9	*2*	*20*
	2	5	*1*	*14*	5	*0*	*13*
	3	4	*0*	*12*	1	*0*	*7*
	mean	**4**	*1*	*8*	**4**	*2*	*9*
ABA	1	408	*182*	*724*	137	*25*	*339*
	2	345	*141*	*640*	380	*164*	*687*
	3	355	*147*	*653*	153	*32*	*363*
	mean	**369**	*235*	*533*	**211**	*113*	*338*
ABA-GE	1	20	*12*	*29*	6	*2*	*12*
	2	19	*11*	*28*	17	*10*	*26*
	3	17	*10*	*26*	10	*5*	*17*
	mean	**19**	*14*	*24*	**11**	*7*	*15*

Superscripts [a] and [b] identify means per genotype per culture type that differ at the $\alpha = 0.05$ level; when there are no such superscripts, the three means did not differ significantly.

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Table 4. Titers of PAs (nmol g^{-1} f.m.) in EM cultures of *P. pinaster* per genotype and culture type: young (1), aged (2) and secondary embryogenic (3). Numbers are back-transformed means and their 95% lower and upper confidence limits (CL).

Genotype	Culture type	Fraction S			Fraction SH			Fraction PH		
		Mean	*95% conf. limit*		Mean	*95% CL*		Mean	*95% CL*	
			Lower	*Upper*		*Lower*	*Upper*		*Lower*	*Upper*
Putrescine										
NM18	1	**264**	*136*	*436*	**317**	*97*	*666*	**64**	*11*	*159*
	2	**291**	*130*	*515*	**114**	*25*	*266*	**71**	*8*	*199*
	3	**386**	*226*	*589*	**219**	*84*	*419*	**62**	*10*	*155*
MM25	1	**452**	*277*	*670*	**94**	*26*	*205*	**22**	*0*	*86*
	2	**724**	*358*	*1219*	**155**	*20*	*418*	**43**	*4*	*124*
Spermidine										
NM18	1	**396**[a]	*229*	*609*	**663**	*305*	*1158*	**97**	*26*	*215*
	2	**747**[b]	*461*	*1101*	**180**	*57*	*371*	**173**	*53*	*361*
	3	**1070**[b]	*780*	*1406*	**363**	*174*	*621*	**164**	*64*	*309*
MM25	1	**47**	*4*	*136*	**36**	*1*	*116*	**5**	*0*	*50*
	2	**192**	*31*	*490*	**33**	*0*	*197*	**11**	*0*	*65*
Spermine										
NM18	1	**69**[a]	*34*	*117*	**175**[a]	*80*	*308*	**24**	*6*	*54*
	2	**231**[b]	*148*	*333*	**24**[b]	*3*	*63*	**49**	*16*	*102*
	3	**204**[b]	*139*	*281*	**108**[a]	*54*	*181*	**38**	*13*	*74*
MM25	1	**66**	*31*	*113*	**0**	*0*	*7*	**3**	*0*	*19*
	2	**43**	*5*	*118*	**1**	*0*	*30*	**1**	*0*	*14*

Means per genotype per culture type followed by the same superscript do not differ at the α = 0.05 level; when there are no such superscripts, none of the means differ significantly.

Table 5. Global DNA methylation levels in *P. pinaster* NM18 and MM25 cultures.

Genotype and culture type	DNA methylation (%)*
NM18-1	19.1
NM18-2	17.8
NM18-3	18.5
MM25-1	18.8
MM25-2	18.6

*standard error = 0.37

Table 6. Mean growth increases of *P. pinaster* (NM18-2a) EM and the global DNA methylation levels on medium with various concentrations of 5-azaC after 9 d of culture and numbers of somatic embryos produced from the cultures after each treatment (Experiment 1), and the global DNA methylation levels after 5-azaC treatment for 14 d (Experiment 2).

	Experiment 1			Experiment 2
5-azaC (µM)	Mean growth (g) (s.d.)*	DNA methylation (%) (s.e.)**	No. of somatic embryos g^{-1} f.m.	DNA methylation (%) (s.e.)**
0	3.7 (0.5)	18.0 (0.98)	3	17.8 (0.78)
5	3.0 (0.4)	18.7 (0.98)	0	18.0 (0.78)
10	2.7 (0.2)	19.5 (0.98)	15	17.9 (0.96)
15	2.6 (0.5)	21.3 (0.98)	10	16.4 (0.78)
20	2.4 (0.3)	19.3 (0.98)	3	16.4 (0.78)
40	2.3 (0.1)	19.3 (0.69)	5	Not determined

*standard deviation

** standard error

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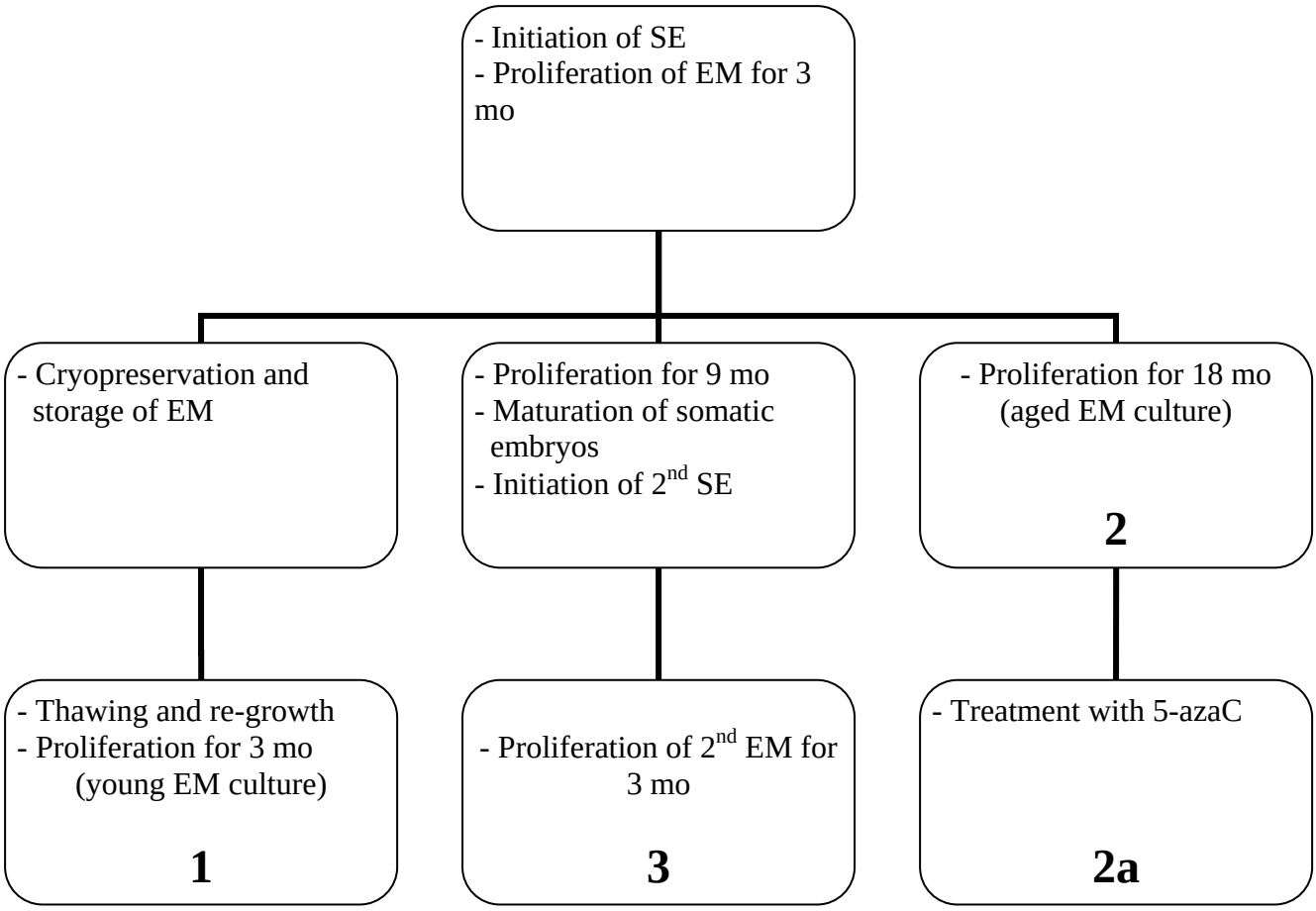
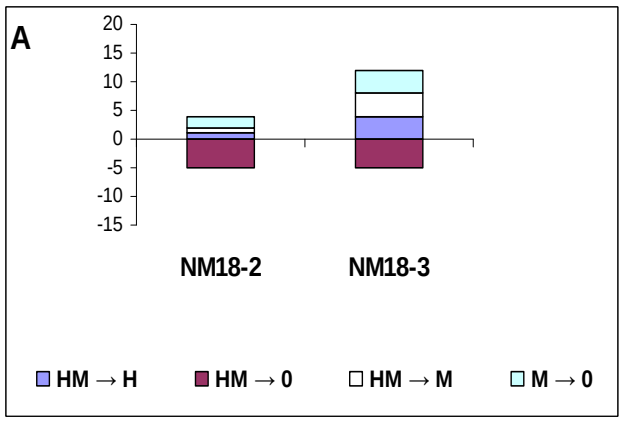
FIGURE LEGENDS

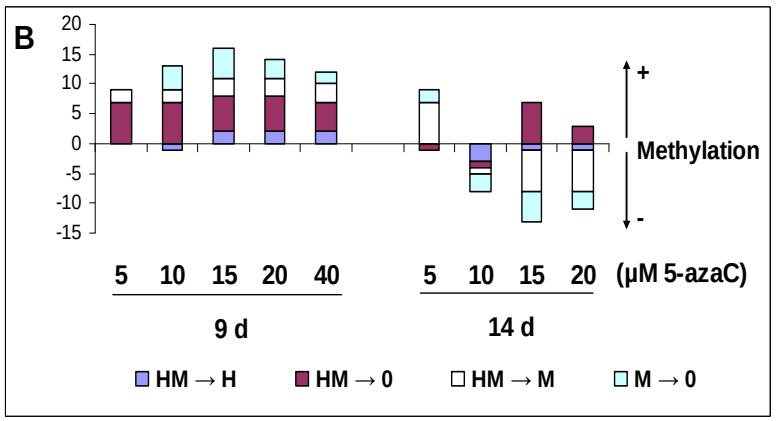
Figure 1. Origin of the *P. pinaster* sampled EM cultures designated 1 (young), 2 (aged), 2a (aged treated with 5-azaC), and 3 (2^{nd} embryogenic tissue) used for the biochemical analysis.

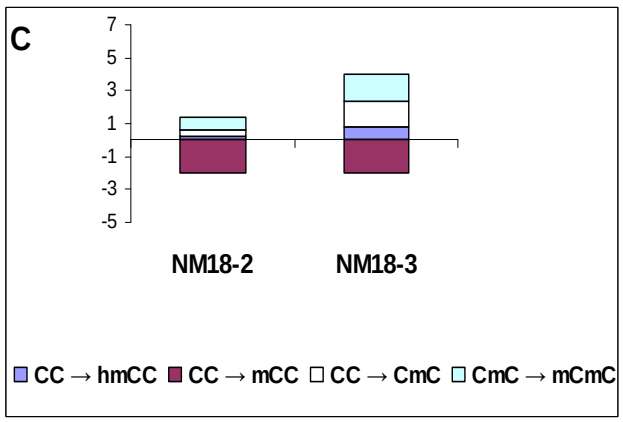
Figure 2. *P. pinaster A*, young (1) and *B*, aged (2) EM cultured on maturation medium for 12 wk. Arrows on *A* indicate mature somatic embryos. Bar = 3.46 cm.

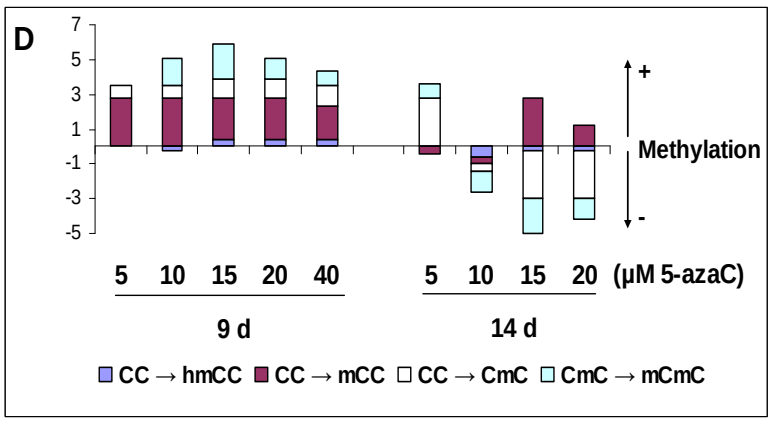
Figure 3. Alterations in DNA methylation detected by MSAP in *P. pinaster*; *A, C* NM18-2 (aged EM culture) and NM18-3 (secondary EM culture) in reference to the methylation status in control NM18-1 (young EM culture) and *B, D* in NM18-2a after 9 and 14 d treatment with 5-azaC compared with non treated control NM18-2; *A, B*, net number of band changes with respect to the controls; *C, D*; percent ratios of the number of specific cytosine single methylation events divided by the number of 5'-CCGG-3' plus 5'-CmCGG-3' quantified in the respective controls.











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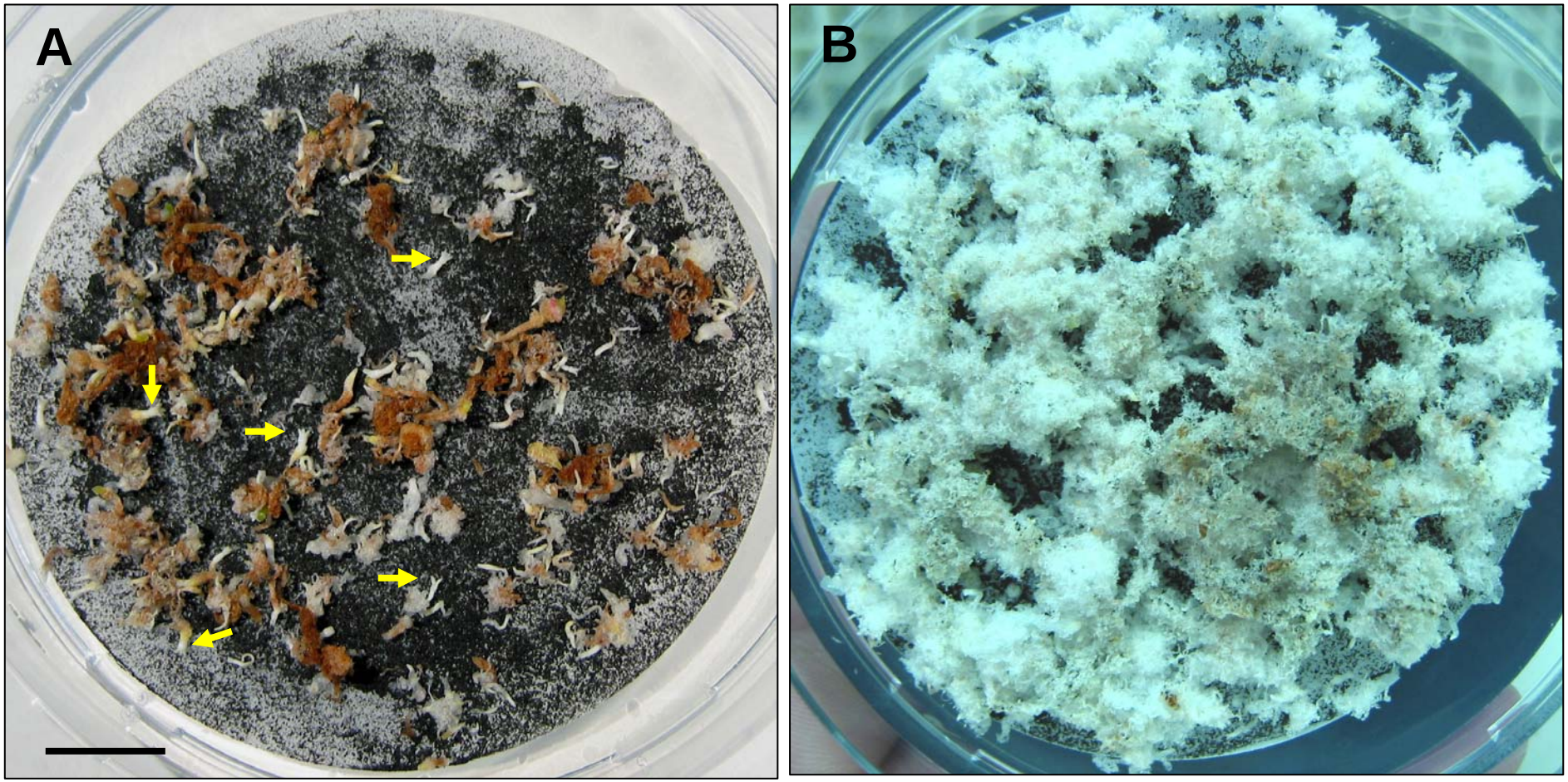

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