Age-dependent biological characteristics of embryonal mass of maritime pine in relation to the embryogenic potential

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Introduction

Somatic embryogenesis (SE) in conifers is at the base of nearly all biotechnology products that potentially might be developed. SE is a powerful tool for clonal propagation that has opened avenues for deployment of superior clonally replicated planting stock in forest plantations (Nehra et al. 2005). Although the SE process can be managed for high efficiency there are several problems that require better understanding of underlying causes. One phenomenon, which occurs after the long-term culture of embryogenic tissue, particularly of pines, is the loss of ability to produce mature somatic embryos and consequently plants (Breton et al. 2005, Lelu-Walter et al. 2006). Such unproductive cultures must be replaced, which is both expensive and undesirable.

The objective of this study was to get an insight into differences between young, primary lines of embryonal mass of maritime pine that produced somatic embryos and the same lines of significantly increased age that stopped producing somatic embryos. We analyzed in both types of material the following:

- hormones and their metabolites
- polyamines
- global DNA methylation levels and pattern
- effect of hypomethylating agents on the embryogenic tissue viability, embryogenic potential and DNA
- methylation levels and patterns.

Material and Methods

In this study we used embryogenic cultures of *Pinus pinaster* (maritime pine) line NM18 of different ages (Fig. 1) and different embryogenic potential (Table 1, Fig. 2).

Fig. 1. Materials used in this study.

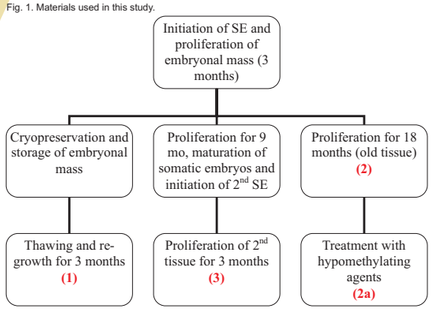


Fig. 2. Embryogenic cultures **(1)** producing somatic embryos and plants and cultures **(2)** that lost their embryogenic potential.

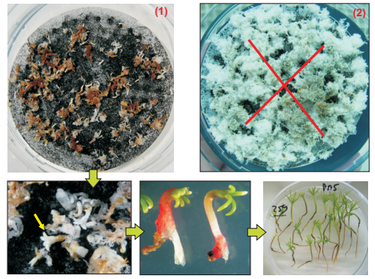


Table 1. Somatic embryo maturation in the young culture (1), old culture (2), and 2nd embryogenic line (3) after 12 to 14 weeks of culture. The tissue was plated at 100 mg f.m. / filter paper (7 cm in diameter) on mLV medium (modified Litvay et al. 1985) with 6% sucrose, 80 uM ABA, and 1% gellan gum.

Material	Number of Petri dishes	Number of mature se g^{-1} fm
NM18 – (1)	6	175
NM18 – (2)	8	0
NM18 – (3)	16	325

Results

Hormones

Table 2. Quantification of hormones and their metabolites in embryogenic cultures of maritime pine according to Jourdain et al. (1997). Auxin (IAA and IAA aspartate), cytokinins (Z and Z riboside, IP and IP adenosine) and abscisic acid (ABA and ABA glucose ester).

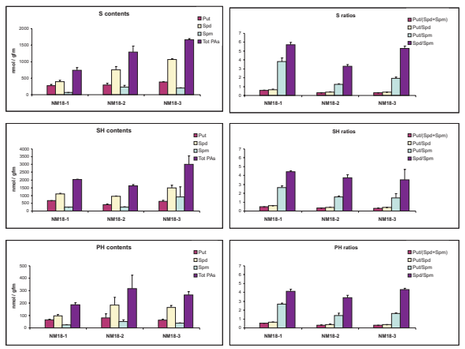
Material	Hormones (pmol g^{-1} d.m.)							
	IAA	IAAasp	Z	ZR	IP	IPA	ABA	ABA-GE
NM18-(1)	4884	1753	355	89	644	89	9280	444
NM18-(2)	2797	755	1066	488	1709	178	8591	422
NM18-(3)	1287	511	333	111	599	89	8281	400

The level of hormones and their metabolites varied in the three types of material (Table 2). In general, in old culture (2) there was a higher level of free cytokinins and their metabolites, whereas the level of IAA was intermediate compared with young embryogenic cultures (1) and (3). ABA and ABA-GE were found to be similar among all the cultures and were at the highest mean concentration compared with the mean levels of auxin and cytokinins.

Polyamines (PAs)

The analysis of PAs, putrescine (Put), spermidine (Spd) and spermine (Spm) and their product of degradation 1,3-diaminopropane (DAP) was carried out in triplicate (25 mg dm / replicate). However, DAP was not detected in any of the tested material. The total content of free PAs was the lowest in the young embryogenic tissue (1) (Fig. 3). The old culture (2) had higher levels of free Spd and Spm compared with cultures (1). In contrast, SH-conjugated PAs were at the higher level in cultures (1) than in cultures (2). On the other hand, the PA contents in PH fraction were similar in both types of material. Generally, all the ratios of PAs in the three fractions were higher in the old culture (2).

Fig. 3. Polyamine profiles



Global DNA methylation

DNA methylation has been associated with numerous biological processes including genomic imprinting, transcriptional regulation, gene silencing and defence of the genome against transposable elements and retroviruses (reviewed by Paszkowski and Whitham 2001). Cytosine methylation analysis in plants has been approached by studying either global levels of methylated cytosines in DNA or by examining the methylation pattern of 5'-CCGG sequences. In the present study we have undertaken both approaches.

Table 3. Global DNA methylation levels in cultures of different ages measured by high performance capillary electrophoresis (HPCE) (Fraga et al. 2000).

Material	DNA methylation (%) (s.d.)
NM18 – (1)	16.6 (0.6)
NM18 – (2)	16.2 (0.7)
NM18 – (3)	15.5 (0.5)

The levels of methylation of the embryonal mass of different ages were from 15.5% (s.d. 0.7) to 16.6% (s.d. 0.7). The 2nd embryogenic tissue appeared to have a slightly lower level of methylation.

Methylation pattern

The methylation pattern was analyzed by methylation sensitive amplification polymorphism (MSAP) using a two-dye IR2 DNA analyzer system (model 4200, LI-COR Biosciences, Lincoln, NE, USA). The digital MSAP gel images were scored using the SAGA^{GT} software Version 3.0 (LI-COR Biosciences) (Fig. 4).

Fig. 4. Examples of changes in the methylation pattern in cultures treated with 15 and 40 µM 5- azaC.

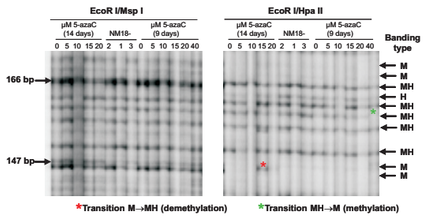
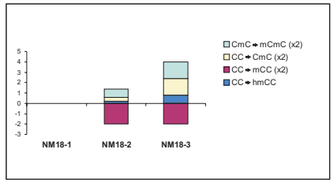


Fig. 5. Ratios of DNA-methylation **changes** with respect to the mean number of CCGG + CmCGG sequences. The results for the cultures (2) and (3) were computed relative to the cultures (1).



Treatment with the hypomethylating agents

Several compounds known to influence the cytosine methylation were tested in the culture medium. These were: 5aza-C, 5aza-dC, zebularine and 3-benzamide. To determine the toxicity level, the cultures (2a) were treated with various concentrations of the above agents for up to 14 days (Fig. 6).

Fig. 6. Toxicity of the hypomethylating agents. Growth of the cultures (fm) is expressed relative to the control, which was set at 100%.

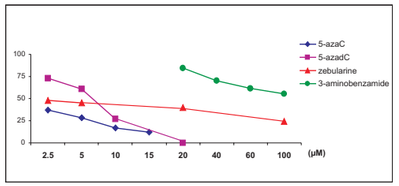
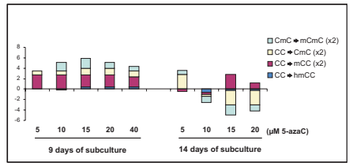


Fig. 7. Ratios of DNA-methylation changes with respect to the mean number of CCGG + CmCGG sequences in control and 5-azaC treated cultures for 9 or 14 days. The changes were computed relative to the control cultures (not treated).



Conclusions

Maritime pine cultures (line NM18) of different ages and embryogenic potential varied with respect to the hormonal and polyamine contents.

The old cultures (2) that lost embryogenic potential were characterized by elevated levels of cytokinins and their metabolites compared with young embryogenic cultures (1). This possibly could explain the high proliferation of the cells and lack of differentiation. Similarly, free PAs were at a higher level in old tissue (2), whereas the SH-conjugated PAs were at a slightly lower level. The ratio of free to SH-conjugated PAs was higher in old cultures, which might coincide with the loss of embryogenic potential.

Global DNA-methylation levels were similar in both types of cultures; however the methylation pattern was different. Aging of the cultures led to DNA-methylation changes (CmC to mCmC, CC to CmC, CC to hmCC) and to de-methylation changes (mCC to CC). The values of the ratios of methylation changes indicated similar amounts of mCC de-methyaltion in both (2) and (3) cultures. However, *de novo* methylation was higher in cultures (3). Treatment with 5-azaC resulted in changes in the methylation patterns. The effect changes on somatic embryo maturation is being tested.

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