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Abstract

Maritime pine is a major species in Europe, especially in France, Portugal and Spain. This species has been subjected to advanced forestry and genetic breeding since the early sixties in France. However, there are strong limitations to genetic betterment of the species by traditional improvement methods because of a long generation time, high genetic load as well as a high genetic redundancy within the breeding population. Sudden and drastic socio-economic and environmental changes in recent years would need a significant paradigm shift in current breeding technology to deliver suitably tested tree varieties in plantation forestry, i.e., there is a need for multi-varietal forestry targeting over a wide range of end-products through various sylvicultural regimes. Field comparison of vegetative propagules is a key towards individual clonal selection and efficient capture of the best genetic stocks. Highly efficient clonal propagation technology is also required for scaling up production of improved varieties. Somatic embryogenesis is considered as the key technology to fulfil such requirements in maritime pine. The species is characterized by increased recalcitrance to vegetative propagation through conventional cuttings as trees are reaching their adult vegetative or reproductive phase. Somatic embryogenesis initiation from mature trees is still challenging in conifers. Therefore, the approach developed for maritime pine is postponed propagation of tested trees by combining somatic embryogenesis initiation from immature zygotic embryos and stable cryopreservation of juvenile embryogenic tissue. This review describes recent achievements and challenges towards efficient

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somatic embryogenesis as a key technology for multi-varietal forestry with maritime pine.

Keywords: Acclimatization; embryo quality; emblings; field test; germination; initiation; maturation; *Pinus pinaster*; zygotic embryo

1. Introduction

Maritime pine (*Pinus pinaster* Ait.) is an industrial heavyweight in Europe, especially in France, Portugal and Spain. More than a quarter of European forestry resources are located in France (1.05 Mha, e-IGN 2015) and this species is accounting each year for more than 25% of the national softwood timber (3.7 Mm^3) and pulpwood (2.8 Mm^3) production. Almost all this production (6.5 Mm^3 in 2013) is obtained from intensively managed plantation forests located in the Aquitania region (0.82 Mha). The mean productivity of this species can be high compared to that of other conifers in France (11.8 m^3/ha/year) and about 70% of the marketed harvest is mechanized. More than 38 000 workers are currently employed in this industry (16.5% of the national forest sector) with an annual turnover of around 2.5 billion Euros (36% as exports, INSEE 2006). « *The number of salaried employees, its role in territorial development and cohesiveness, its large contribution to Aquitania's GDP confer to the maritime pine market a leading part in socio-economic development* » (translated quote, Regional Aquitania Council). Two heavy storms recently affected the Aquitania forest (1999 and 2009) and resulted in ca. 300 000 ha being completely cleared, i.e., 30% of the resource plantations were down and 61 Mm^3 of wood (6 years harvesting) were undervalued (IFN 2009). Rapid reforestation, together with increasing forest resilience to major biotic and abiotic risks are thus major objectives for the forthcoming decades (GIP EcoFor, 2010, www.gip-ecofor.org). If harvesting is maintained at up to 95% of the normal annual growth of the maritime pine forest, as observed before the 2009 storm, a severe maritime pine resource shortage is anticipated by 2020.

In this context, the interest for genetically improved varieties has been considerably reinforced in France and there is a high need for improved seeds (ca. 3t/year). As an indicator of the current reforestation program, the production of seedling plants (>90% improved varieties) reached ca. 45 million in 2013 and 2014. French breeding programs launched in the early sixties by FCBA and INRA were federated in 1995 in a joined initiative called “Maritime Pine for the Future” and involved all other major forest actors in France (CPFA, CRPF, ONF). Up to 15% genetic gain was achieved for volume and straightness in the first and second generation varieties (Figure 1). The rather long generation time of this species (15 years) resulted in significant inertia of the conventional breeding program.

Optimizing genetic gain per breeding selection cycle, taking also into consideration

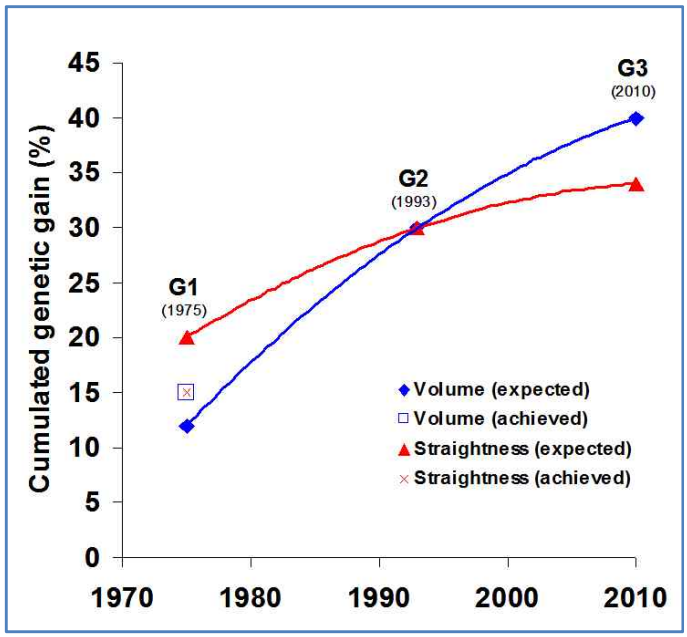

Figure 1. *Cumulated genetic gain (%) expected from first- (G1), second- (G2) or third-generation varieties in maritime pine. Data are expected or achieved genetic gain at age 15 years for growth volume and stem straightness (from Alazard and Raffin 2002). The production of seed orchards started at the date indicated in brackets.*

genetic diversity, is now considered as a requisite for new variety design and deployment. Full productivity of improved-seed orchards for commercial purposes is currently achieved every 17-18 years (Figure 1) and this turnover time is now expected to decrease as a result of improved growth of selected genitors (4^{th} generation expected by 2020). Nevertheless lower genetic gains (10%) are expected by the third round of selection (Figure 1) owing to genetic redundancy within breeding populations. The genetic base of the 4^{th} seed orchard generation has been considerably enlarged to take this phenomenon into consideration (Alazard and Raffin 2002), but sudden and drastic changes in market requirements and environmental constraints in recent years (climate change, abiotic stresses, pests) would need a significant paradigm shift in current breeding technology to deliver suitable tested tree varieties in plantation forestry, i.e., multi-varietal forestry has to be targeted over a wide range of end-products through various sylvicultural regimes. Multi-varietal forestry is simply defined as the deployment of tested tree varieties in plantation forestry (Park 2004; see also Klimaszewska et al. 2007).

Biotechnology offers new opportunities for species improvement, i.e., variety design, conservation and deployment. Advances in genomic- and/or clonally-assisted selection of elite trees would facilitate efficient capture of the best genetic stocks by promoting individual vs. familial selection, i.e., increased selection efficiency of elite genotypes in breeding populations. This can be achieved through accurate and early detection of genes (marker-assisted selection) or genome-wide markers (genomic selection) associated to valuable traits, as well as through field comparison of vegetative propagules (clonal tests). Genomics and particularly genomic selection offers considerable advantages for breeding forest trees over the next decades (Plomion et al. 2015). Efficient clonal propagation would also greatly facilitate deployment of selected varieties. Synergies are, therefore, expected between conventional breeding, early selection and powerful methods for clonal propagation of elite genotypes to implement innovative, multi-varietal forestry in conifers (El-Kassaby and Klápště 2015). Optimized and balanced genetic gain and diversity together with greater flexibility in variety deployment are expected from multi-varietal forestry in pines (Weng et al. 2011; Klimaszewska et al. 2007).

With regard to maritime pine, effective vegetative propagation through conventional cuttings or *in vitro* micropropagation for selection among trees in their adult vegetative or reproductive phase, proved to be difficult to achieve and/or too expensive for being implemented into breeding programs (Trontin et al. 2004; De Diego et al. 2008). In contrast somatic embryogenesis has promising attributes to scale up production of improved varieties in conifers (reviewed in Lelu-Walter et al. 2013; Klimaszewska et al. 2015), particularly in pines (Klimaszewska et al. 2007). Somatic embryogenesis initiation from vegetative explants of adult selected trees has still to be demonstrated for maritime pine as well as for other conifers (see Trontin et al. in this book). Therefore, the ongoing strategy developed for maritime pine throughout Europe involved the postponed propagation of tested trees. This involves large scale regeneration of plants from embryonal masses (EMs) that have been cryopreserved in juvenile form, i.e., these masses were initiated from dissected zygotic embryo (ZE) from immature seed. Somatic embryogenesis initiation was first reported for maritime pine by Jarlet-Hugues (1989) with some insights into both EM initiation and multiplication by Bercetche and Pâques (1995). Full plant regeneration from propagated EM became effective 10 years later (Lelu et al. 1999). Extensive research to improve this process has since been undertaken mainly in France (Ramarosandratana et al. 2001a,b; Jordy and Favre 2003; Breton et al. 2005, 2006; Lelu-Walter et al. 2006; Park et al. 2006; Pérez-Rodríguez et al. 2006; Klimaszewska et al. 2009; Trontin et al. 2011; Morel et al. 2014a,b), but also in Portugal (Miguel et al. 2004; Marum et al. 2009a) and in Spain (Humánez et al. 2012; Álvarez et al. 2013). A synthesis of methodological and scientific aspects of progress up to 2005-2006 can be found in Harvengt (2005)

and Klimaszewska et al. (2007). After more than 20 years of continuous effort, the technology has been sufficiently refined to allow high genotype capture at the initiation step (77%, Park et al. 2006) and to achieve production of somatic embryos (SE) and somatic seedlings (emblings) that have been tested in field tests at FCBA for more than 15 years. Implementation of the technology into multi-varietal forestry would require both high-quality cotyledonary SEs similar to the seedling standard and cost-effective solutions for industrial application of somatic embryogenesis. In this chapter we present recent and major achievements as well as prospects towards procuring high-quality somatic seedlings of maritime pine, particularly at the induction and maturation steps. We also highlight the useful and practical issue of combining somatic embryogenesis with new genomics applications, reverse genetics and cryopreservation. We finally report on some of our first results from field tests indicating that application of somatic embryogenesis in association with both breeding (clonal and varietal tests) and variety deployment should be profitable to the economy of maritime pine forestry.

2. Somatic embryogenesis as a critical enabling biotechnology in maritime pine

Somatic embryogenesis is the core technology in maritime pine to support cryopreservation of genetic resources and genetic, epigenetic, and reverse genetic studies through genetic transformation. It has recently been demonstrated that somatic embryogenesis provides a true *in vitro* model that mimics zygotic embryo development in maritime pine up to the cotyledonary stage (Morel et al. 2014b).

2.1 Somatic embryogenesis vs. reverse genetics

Reverse genetics, defined as ectopic expression or silencing of candidate genes in selected genotypes, has become an indispensable research tool for functional dissection of traits of interest in forest trees. In maritime pine as in other conifers, a long generation time and long life span, high genetic loads as well as high genetic redundancy, are major obstacles to follow standard genetic practices, including association genetics. Validating marker associations with specific properties before transfer into breeding selection models is still challenging. Stable *Agrobacterium*-mediated genetic transformation of maritime pine EMs and transgenic somatic plant regeneration was first reported by Trontin et al. (2002) and further developed in both France (FCBA/INRA collaboration) and at IBET/ITQB in Portugal (reviewed in Trontin et al. 2007). Very similar protocols were also recently developed in Spain (Álvarez and Ordás 2013). The common protocol used by FCBA/INRA has been sufficiently refined to envisage practical application in reverse genetics as an attractive complement to association studies.

The method is based on phosphinothricin selection of reference genotype and has been transferred to 5 teams in Europe (Universities of Málaga, Alcalá, and Valencia in Spain; IBET/ITQB in Portugal; Humboldt University of Berlin in Germany, Trontin et al. 2013) in support of the multinational "Sustainpine" project (http://www.scbi.uma.es/sustainpine/). The maritime pine toolbox for genetic transformation has been further improved by this project. This is one of the greatest efforts worldwide for gene functional analysis in conifers. Transgenic embryogenic lines and somatic plants are currently being investigated for various overexpression and/or RNAi constructs (ihpRNA strategy, intron-spliced hairpin RNA), targeting 39 genes involved in wood formation, carbon and nitrogen metabolisms, ammonium regulation, stress resistance (drought and nutrition) as well as embryogenesis and plant development (de Vega-Bartol et al. 2013a; Hassani et al. 2013; Carneros et al. 2014; Mendoza-Poudereux et al. 2014; Trontin et al. 2014).

2.2 Somatic embryogenesis vs. genomics

In the context of climate change and induced biotic and abiotic stresses, genomics for forest trees is developing rapidly, showing significant achievements and providing new perspectives for breeding (Plomion et al. 2015). In maritime pine much is expected from the full genome sequence that should be available at the end of the European project "ProCoGen" (http://www.procogen.eu/). A reference transcriptome has already been established (Canales et al. 2014) from various tree sources, experimental conditions and tissues, including EMs and cotyledonary SEs. This study provided a large catalogue of more than 26 000 unique transcripts and also a collection of 9641 full-length cDNAs (FLcDNAs). As stated above, the large availability of FLcDNAs paved the way for the collaborative, multinational application of reverse genetics towards functional dissection of traits of economic and ecological interest in maritime pine. Embryogenesis-related genes are among key genes for future application in tree improvement and new variety deployment in conifers (Plomion et al. 2015; detailed review in Trontin et al. 2015). Reference transcriptome (Canales et al. 2014) but also genome-wide transcriptomics (de Vega-Bartol et al. 2013b; Morel et al. 2014a) and proteomic profiling (Morel et al. 2014a,b) of both SEs and ZEs have already provided significant clues for a better knowledge of the molecular aspects of embryo development. In particular, specific genes and master regulators such as transcription factors and genes involved in the epigenetic complex for regulation of gene expression have been found expressed in maritime pine (Gonçalves et al. 2007; de Vega-Bartol et al. 2013b; Morel et al. 2014a). This complex and still fragmented knowledge started to deliver accurate marker genes and new molecular tools to refine somatic embryogenesis in conifers and check for (epi)somaclonal variation throughout the process (reviewed in Miguel et al., in this book). Maritime

pine expression studies of *glutamine synthase* (*GS*) isoform genes, expressed in either photosynthetic (*GS1a*) or vascular tissue (*GS1b*), indicated that these genes could serve as indicators of early differentiation of procambial cells (*GS1b*) or abnormal, early germination of cotyledonary SEs (*GS1a*) that apparently did not reach full maturity (Pérez-Rodríguez et al. 2006). Similarly, in a combined transcriptomic and proteomic study of the molecular responses promoting SE development on maturation medium, Morel et al. (2014a) proposed that *germin-like protein* and *ubiquitin-protein ligase* genes could be used as predictive markers of embryo development (see below part 4) as early as after 1 week on maturation medium (out of 12 weeks to complete maturation). Marker genes could be of great interest to avoid unnecessary expenses associated with sub-optimal culture conditions.

2.3 Somatic embryogenesis vs. cryopreservation

Crypreservation of initiated embryogenic lines is effective in maritime pine using either a programmable freezer with slow cooling (Harvengt 2005) or Nalgene Cryo 1°C Freezing Containers (Marum et al. 2004; Lelu-Walter et al. 2006). Density of the embryogenic suspension was shown to affect recovery of cryopreserved lines and the optimal concentration was estimated to be 250 mg mL^{-1} (Marum et al. 2004). Pre-treatment of the embryogenic suspension is performed in presence of either sucrose 0.5 M (Harvengt 2005), maltose 0.4 M (Marum et al. 2004) or sorbitol 0.4 M (Lelu-Walter et al. 2006). In general a cryoprotectant such as DSMO (dimethyl sulfoxide) is added to the suspension at a final concentration in the range 5-7.5%. Recovery of cryopreserved lines was improved when DMSO was combined with polyethylene glycol (PEG 10%, Marum et al. 2004). A simplified, cost effective method has been developed at INRA (Lelu-Walter et al. 2006). The method involved a single-step pre-treatment of EM with sorbitol and DMSO, slow cooling with no need for a programmable freezer and rapid recovery on filter paper discs. At FCBA a cryo-collection of over 1850 embryogenic lines has been established from 94 families, especially 17 elite families from the FCBA/INRA breeding program developed within the framework of the joined initiative “Maritime Pine for the Future”. The first lines were cryopreserved in the late 1990s and are still used for experiments without any apparent loss in regenerative capacity nor evidence for any (epi)somaclonal variation related to the cryopreservation process. However, a systematic loss of maturation ability within 6 months post reactivation from the cryopreserved stock is observed (Breton et al. 2006). At FCBA virtually all lines are easily recovered from the cryopreserved stock within 2-4 weeks.

3. Somatic embryogenesis in *Pinus pinaster*

3.1 Induction step

Somatic embryogenesis initiation is currently achieved from immature ZEs, either dissected or kept in place within the whole megagametophyte (Table 1). Using dissected immature ZEs allows to choose their developmental stage for culture more carefully. The ZE reaches its highest capacity for initiating somatic embryogenesis in the early stages of late embryogeny, i.e., when it attains dominance and up to the precotyledonary stage. Interestingly, secondary somatic embryogenesis has also been successfully initiated from cotyledonary SEs at high frequency (up to 82%, Klimaszewska et al. 2009). In contrast, the embryogenic potential of ZEs significantly decreases at the early cotyledonary and cotyledonary stages, and fully mature ZEs are no longer responsive in maritime pine as in most other pine species. Similarly somatic embryogenesis from older maritime pine trees in their adult vegetative or reproductive phase remains challenging. Some recent and promising results suggested that EM-like tissue could be obtained from primordial shoot bud explants from mature trees up to 34 years old but these findings need to be further investigated (see Trontin et al. in this book).

Table 1. *Initiation rate of somatic embryogenesis in P. pinaster from different explants and developmental stages.*

Explant type and dev. stage	Seed origin	Basal medium	PGR type	Initiation response (% range)	References
Dissected ZE					
Pre cotyledonary	3 open pollinated trees	mLV	2,4D+BA	26-40	Lelu et al. (1999)
		mLV	No PGR	0-13	
	9 full-sib families	mLV	2,4D+BA	65-100	Lelu-Walter et al. (2006)
	4 open pollinated + 6 controlled crosses	mLV	CPPU	56-75	Park et al. (2006)
Un-dissected ZE					
Dominant ZE	5 open pollinated trees	mLV	2,4D+BA	0-82	Humanez et al. (2012)
Pre cotyledonary	20 open pollinated trees	DCR	2,4D+BA	5-49	Miguel et al. (2004)
Isolated SE					
Cotyledonary	5 embryogenic lines	mLV	2,4D+BA	13-82	Klimaszewska et al. (2009)

BA: benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; PGR: plant growth regulator; SE: somatic embryo; ZE: zygotic embryo

Two different basal media are commonly used at the induction step depending on the laboratory and the plant material origin or culture conditions. Open-pollinated seed families from Portugal had the highest initiation rate (5-49%, Miguel et al. 2004) on basal DCR medium (Gupta and Durzan 1985). Similarly, in

France, a modified DCR basal medium (mDCR, Breton et al. 2005) containing DCR macroelements and Murashige and Skoog (1962) micronutrients was routinely used to initiate somatic embryogenesis from 31 full-sib and 9 open-pollinated seed families but at a quite low mean initiation rate of 23.3% (Figure 2). In contrast full-sib families from INRA, France (Lelu-Walter et al. 2006) and open-pollinated seed families from Spain (Humánez et al. 2012) had the highest initiation frequencies (82-100%) on a modified Litvay medium (Litvay et al. 1985) that contained half-strength macroelements except iron and EDTA (mLV, Klimaszewska et al. 2000). A comparison of both mDCR and mLV media has been undertaken at FCBA. Considering a large selection of full-sib and open-pollinated families, mLV gave consistently higher initiation rates than mDCR over various initiation efforts from 2000 to 2009 (67.5 vs 23.3%, Figure 2).

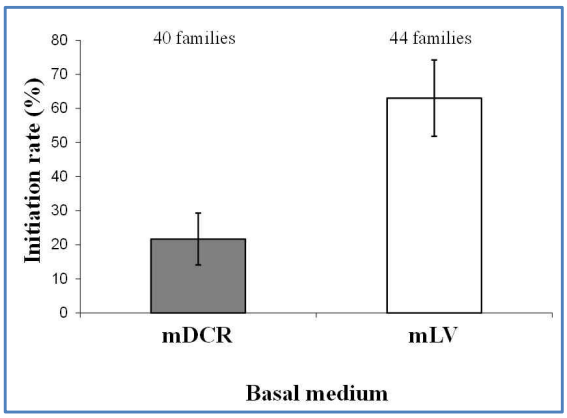

Figure 2. *Initiation rate of somatic embryogenesis from various controlled- and open-seed families in maritime pine as a function of basal medium: mDCR (modified from Gupta and Durzan 1985) and mLV (modified from Litvay et al. 1985). Bars represent 95% confidence limits. Data have been computed from various initiation efforts at FCBA from 2000 to 2009. N = 13758 (mDCR) or 8841 (mLV).*

Most induction media are supplemented with a combination of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at 9.0-13.5 µM and the cytokinin benzyladenine (BA) at 2.2-4.4 µM (Miguel et al. 2004; Harvengt 2005; Lelu-Walter et al. 2006; Humánez et al. 2012). Somatic embryogenesis could also be initiated without plant growth regulator (PGR) but the initiation frequency remained comparatively low (0-13%, Lelu et al. 1999). More recently, induction medium was supplemented with the phenylurea CPPU (a potent cytokinin) at 4.0 µM instead of 2,4-D and BA. In combination with Litvay basal salts (mLV), CPPU resulted in significantly improved mean initiation rates compared to the one obtained with 2,4-D and BA (77 *vs.* 34%, Park et al. 2006). This result was

confirmed and strengthened during subsequent initiation efforts at FCBA in 2005, 2006 (Figure 3) and 2007, 2008 (Figure 4). Different mLV-based media and control mDCR were supplemented with various combinations and concentrations of PGR, either 2,4-D and BA or CPPU (Table 2).

***Table 2**. Formulation of mLV- and mDCR-based media used for initiation experiments in P. pinaster at FCBA: fixed and variable components (see Figure 3, 4).*

			Traces (mg l^{-1})		PGR (µM)								
Basal media[a]	Vitamins	Sucrose (g l^{-1})	$NiCl_2$	$CoCl_2$	No PGR	2,4-D 2.2 BA 2.3	2,4-D 9.0 BA 4.4	2,4-D 13.5 BA 2.2	CPPU 0.5	CPPU 1	CPPU 2	CPPU 4	CPPU 6
mLV	LV 1X	30	0	0.125		LV3	LV1,2[b]	LV5				LV4	
mLV	LV 10X	10	0	0.125	LV6,7[b]		LV16		LV8	LV9	LV10	LV11, 12, 14[b]	LV13
mLV	LV 1X	1		0.125								LV17	
mLV	LV 10X	10	0.72	0.125								LV15	
mDCR	DCR 1X	30	0	0.025	DCR1,2[b]			DCR3,4[b]					
mDCR	DCR 1X	30	0.72	0.125								DCR6	

[a]Fixed components; mLV modified from Litvay et al. (1985) = macro LV 0.5X, micro LV 1X, 100 mg l^{-1} meso-inositol, 1 g l^{-1} casein hydrolysate, 0.5 g l^{-1} glutamine, pH 5.8; mDCR modified from Gupta and Durzan (1985) = macro DCR 1X, micro MS 1X, 100 mg l^{-1} meso-inositol, 0.5 g l^{-1} casein hydrolysate, 0.25 g l^{-1} glutamine, pH 5.8; [b]Same medium but different initiation procedures

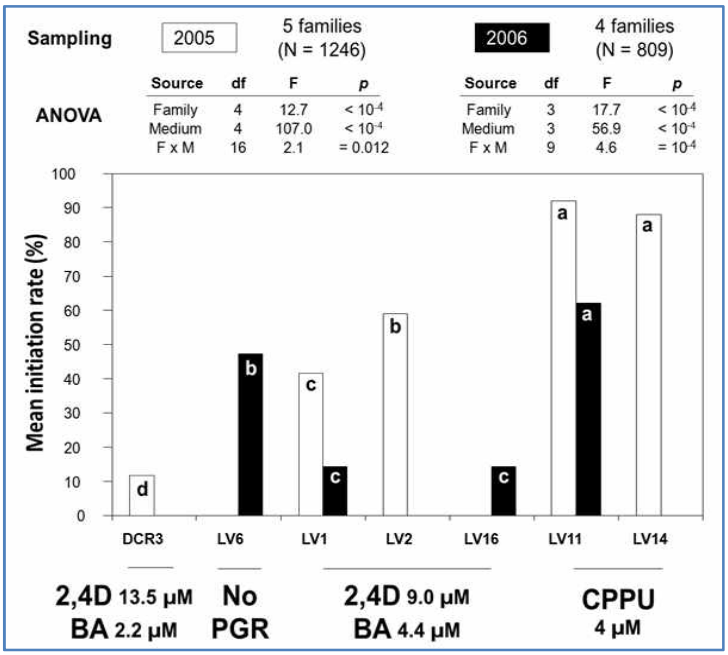

***Figure 3.** Mean initiation rate (%) of somatic embryogenesis in maritime pine with different mLV-based media (LV1,2,6,11,14,16, see Table 2) or control mDCR media (DCR3, Table 2) supplemented with different combinations of PGR, either 2,4-D/BA or CPPU. Sampling and ANOVA are indicated for each initiation effort (2005, 2006) involving 4-5 families. For each year, significant variations between means (SNK tests, α=0.05) are indicated by different letters.*

The "Family" and "Medium" effects were always significant (ANOVA) together with the interaction "Family x Medium" (excepted in 2007). Up to 92% mean initiation rates (2005, Figure 3) were obtained using the mLV/CPPU (4.0 µM) medium with only moderate variation between trial years (62-92%, Figures 3, 4). The optimal CPPU concentration was determined (Figure 4) and it was found that an up to fourfold reduction of the original dose (1.0 µM vs. 4.0 µM used by Park et al. 2006) could be used without any significant decrease in initiation rate.

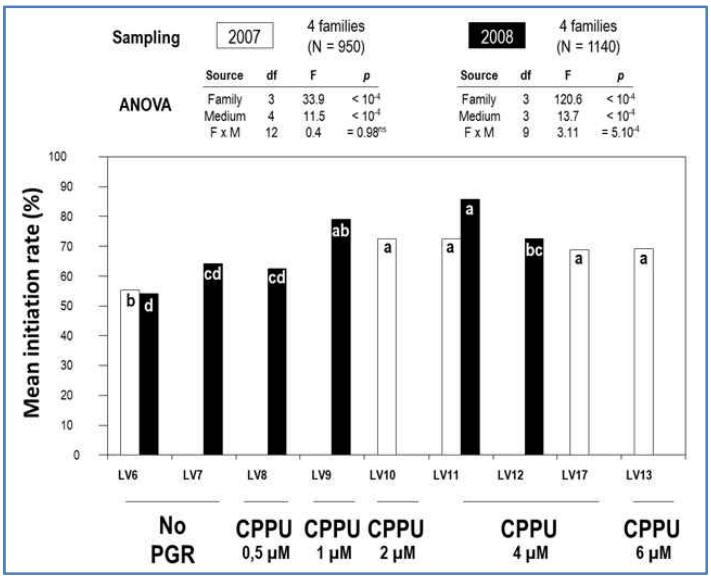

Figure 4. *Mean initiation rate (%) of somatic embryogenesis in maritime pine with different mLV-based media (LV6-13, 17, see Table 2) supplemented with increasing concentration of CPPU. Sampling and ANOVA are indicated for each initiation effort (2007, 2008) involving 4 families. For each year, significant variations between means (SNK tests, α=0.05) are indicated by different letters.*

Interestingly, high initiation rates were also obtained in the case of some families by reducing the exposure time of dissected ZEs on mLV/CPPU (4.0 µM) from 8 weeks (LV11 medium) to only one week (LV12, Figure 4). Initiation rates remained high (47-55%, Figures 3, 4) when initiation media were deprived of CPPU, thus confirming the usefulness of mLV for somatic embryogenesis initiation in maritime pine compared to mDCR. Substituting CPPU (4.0 µM) for the 2,4-D (9.0 µM) and BA (4.4 µM) PGRs in combination with 10 (LV16) or 30 g L^{-1} sucrose (LV1, LV2) significantly reduced the initiation rate (Figure 3). In contrast, CPPU did not have any positive effect in combination with mDCR-based media (data not shown).

In conclusion, CPPU significantly increased the initiation rate to reach a maximum of 65-85% in the range 1-4 µM. We concluded that CPPU at 1 µM was optimal in maritime pine. Family effect was confirmed to be highly significant but

with mean genotype capture within the family of 77%, the variation among genetic backgrounds is now established within acceptable limits and has huge practical implications in breeding programs. Investigation of 3 more families during the FCBA 2009 initiation effort suggested that the mean initiation rate can be further significantly increased by introducing a subculture step of immature explants on induction medium. A 86% mean initiation rate was obtained with a genotype capture rate between 68 and 96% (data not shown).

3.2 EM proliferation and genetic stability

Once obtained, EMs are transferred onto maintenance medium and subcultured weekly or biweekly in clumps onto fresh medium in order to sustain their proliferation. Both maturation yield and SE quality decreased as a function of EM subculture number in maritime pine (Breton et al. 2006; Trontin et al. 2011). Concomitantly, morphological degradation of immature embryos occurred suggesting a progressive loss of regenerative capacity of embryogenic lines. Breton et al. (2005) showed that the aging effect can be lowered by using a high subculture frequency of clumps (7 days instead of 14 days). For rapid EM amplification prior to the maturation treatment, the plating method previously described by

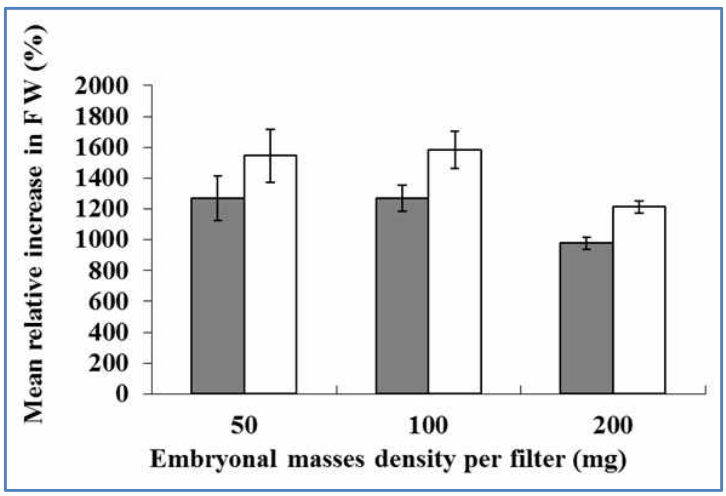

Figure 5. *Mean relative increase in fresh biomass of maritime pine embryonal masses as a function of the density per filter and basal medium during a two-week subculture period.* ▭ *mDCR (modified from Gupta and Durzan 1985);* ▭ *mLV (modified from Litvay et al. 1985). Bars represent 95% confidence limits. FW: fresh weight.*

Klimaszewska and Smith (1997) for *P. strobus* has been adapted for maritime pine (Lelu et al. 1999). EMs dispersed in liquid medium are poured over a filter paper

disc and subcultured every two weeks. A low EMs density of 50-100 mg per filter as inoculum was found optimal (Figure 5). Here again Litvay-based media significantly improved EM growth on filter paper at different cell densities compared to on mDCR (Figure 5). An up to 1500% mean relative increase in biomass can routinely be obtained upon biweekly subculture.

EMs morphology and cytology have been characterized in relation to their proliferation and maturation ability (Ramarosandratana et al. 2001b; Breton et al. 2005). Two different EM morphotypes have been defined, i.e., as smooth or spiky. The spiky morphotype is characterized by the presence of early SEs protruding from the periphery of the EM (Ramarosandratana et al. 2001a) and was associated with increased biomass production (Breton et al. 2005). Proliferation of EMs was also found to be strongly affected by genotype (variation among cell lines) and subculture frequency. During proliferation, microscopic changes of EM occurred, leading to a low occurrence of the early SEs stages, concomitant with a gradual increase in growth rate. EM growth on proliferation medium and early SE development were found to be disconnected. In order to preserve a spiky morphotype, i.e., to retain early embryogenic ability during proliferation, EM should be sub-cultured weekly for a short period (less than 6 months) on a maltose-containing medium without PGRs (Breton et al. 2005). Maltose was found to increase the frequency of well-developed early SE during proliferation.

No major change in ploïdy level was detected in proliferating EM or during early embryo development using flow cytometry (Marum et al. 2009a). In contrast, genetic variation was detected at seven microsatellite loci (SSRs) after prolonged EM subculture for up to 22 months as well as in emblings regenerated from cotyledonary embryos (Marum et al. 2009b). However genetic stability at the analyzed loci could not be associated to abnormal development of emblings.

3.3 EM maturation ability and SE development

Cotyledonary SEs development from embryogenic cultures is under strong genetic control in maritime pine with both maternal and paternal significant effects (Lelu-Walter *et al.* 2006). However, maturation conditions were improved to the point where the variation in cotyledonary SE yield from different genetic backgrounds remained within acceptable limits. The effect of PEG in maturation medium is not clearly established in maritime pine with either no effect (Ramarosandratana et al. 2001a) or a positive effect (Miguel et al 2004). Significant progress was obtained using Litvay-based instead of DCR-based maturation media (Table 3). Mean maturation yield calculated from a large sample of embryogenic lines (238 lines/year on average) matured at FCBA from 2000 to 2009 was 50.5±2.3 SE g^{-1} FW when cultured on mLV, as compared with only

Table 3. *Somatic embryo yield obtained in P. pinaster as a function of different formulations of DCR- and Litvay-based maturation medium.*

Tested lines		Basal	ABA	Sucrose	Gellan	SE yield	Reference
Nb	Maturing[a]	medium[b]	(µM)	(M) / PEG ($g\ l^{-1}$)	gum (g)	g^{-1} FW	
5	5 (100%)	DCR	80	0.17/0	9	8-99	Ramarosandratana et al. (2001a)
896	108 (12%)	DCR	120	0.06/100	10	na	Miguel et al. (2004)
18	18 (100%)	mLV	80	0.2/0	10	2-441	Lelu-Walter et al. (2006)
39	32 (82%)	mLV	80	0.2/0	9	0-192	Trontin et al. (2011)
26	15 (58%)	mLV	80	0.2/0	10	0-274	Hùmanez et al. (2012)

[a]Giving rise to cotyledonary somatic embryos. FW: fresh weight; na: not available

[b]DCR: Gupta and Durzan (1985); mLV: modified from Litvay et al. (1985).

ABA: abscisic acid; PEG: polyethylene glycol; SE: somatic embryo; FW: fresh weight

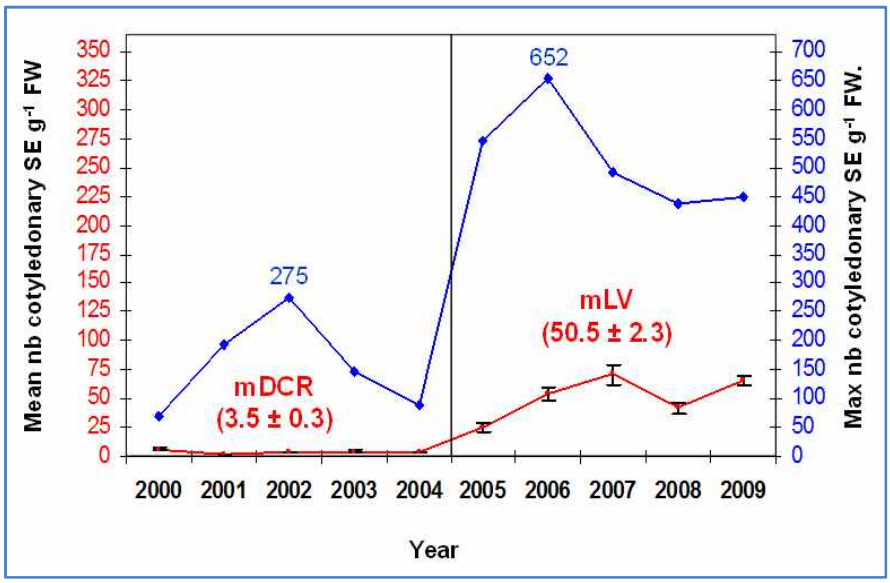

Figure 6. *Maturation ability of maritime pine embryogenic lines as a function of basal medium over 10 years experiments with mDCR (2000-2004) and mLV (2005-2009). Both mean (red curve) and maximum yields (blue curve) in cotyledonary SEs are shown. A mean of 238 lines were investigated per year (18-691 lines). mLV basal medium started to be routinely used in 2005 at FCBA following the results by Park et al. (2006).*
mDCR: modified DCR medium from Gupta and Durzan (1985); mLV: modified LV medium from Litvay et al. (1985). Bars for mean cotyledonary SE yield represent 95% confidence limits. FW: fresh weight.

3.5±.3 SE g^{-1} FW on mDCR (Figure 6). This is a 14-fold increase in mean maturation ability. We also observed a 5-fold increase in mean maximum yield of individual lines from ca. 100 to 500 SE g^{-1} FW on mLV. Changing mDCR for mLV formulations significantly improved genotype capture and performances at

the maturation step. In a recent study of 39 lines from 4 elite families, the genotype capture, calculated as the frequency of lines producing at least 50 cotyledonary SE g^{-1} FW, was estimated to be 43.6% in lines propagated for 18 weeks of subculture (Trontin et al. 2011). However, 10 weeks of subculture later, the genotype capture was strongly reduced to a low 12.8%. An additional consequence of line aging as a function of subculture number was a significant decrease in the size of cotyledonary SEs as measured by embryo or hypocotyl length (Breton et al. 2006). This effect was observed on both mDCR and mLV basal media. As a result, embryo quality significantly decreased, resulting in a lower germination ability (FCBA, unpublished).

As a low genotype capture and reduced SE quality are serious drawbacks for development of somatic embryogenesis at acceptable cost, these results are further evidencing for the need to carefully process both cryopreserved stocks and propagation of embryogenic lines after reactivation from the cryopreserved stock.

Future research in maritime pine should, therefore, focus on achieving a better understanding of the physiological and molecular mechanisms that cause the loss of competence of embryogenic lines to form cotyledonary SEs, resulting in a strong reduction of both maturation yield and genotype capture at this crucial step. Klimaszewska et al. (2009) investigated the young, 3-month-old (productive), and aged, 18-month-old (non-productive) versions of the same embryogenic line (two genotypes tested) with regard to the levels of hormonal and polyamine profiles as well as global and specific (MSAP, methylation-sensitive amplification polymorphism) DNA methylation patterns. Inconsistent hormonal and polyamine profiles were observed in EM cultures of similar phenotypes. Furthermore, the global DNA methylation level measured as the percent of methylated cytosine (% mC) did not significantly change between young and aged cultures (17.8-19.1% mC). Similarly, global DNA methylation was found to be genotype dependent but could not be associated with maturation ability of 3 embryogenic lines with contrasted performances, with the % mC being 12.9% for the line with high maturation ability, 8.5% for the medium and 15.2% for the line with low maturation ability (FCBA, unpublished results).

Treatment of an aged culture with the DNA hypomethylating drug 5-azacytidine (5-azaC) at 10-15 µM greatly reduced growth (cytotoxic effect) but slightly improved EM maturation ability (from 3 to 10-15 cotyledonary SE g^{-1}) suggesting that there is an epigenetic-related origin of the progressive loss of regenerative capacity (Klimaszewska et al. 2009). MSAP profiling of young and aged lines further supported the idea that aging is associated to epigenetic DNA changes (qualitative alterations) involving net demethylation at specific target sequences concomitant with methylation at other sites. A net DNA methylation increase was detected in EM samples of an aged line treated with 5-40 µM 5-azaC for 9 days. In contrast net DNA demethylation was observed in samples subjected

to longer exposure (14 days) at similar 5-azaC concentration, suggesting early cytotoxic and late hypomethylating effects. The consequence of these 5-azaC-induced changes onto the viability and maturation potential of treated tissue remains to be investigated. Overall it suggests that some modulation of DNA methylation could restore the maturation ability. Interestingly this study revealed that secondary EM cultures induced from cotyledonary SEs have better maturation ability than aged EM cultures. It would be interesting to investigate the DNA methylation pattern of these secondary EMs. Secondary somatic embryogenesis appears to be a useful tool to restore maturation ability of embryogenic lines in maritime pine and could also help manage the cryopreserved stock. As stated in the above section, various cultural practices could be used to slow down the aging process, including the use of maltose-based and PGR-free medium and high subculture frequency (Breton et al. 2005). Again it would be interesting to check the DNA methylation pattern of tissues exposed to these cultures conditions.

3.4 SE germination, acclimatization and plant recovery (SE conversion to emblings)

Cotyledonary SEs usually germinated at high frequencies (73% on average Table 4), up to 100% for Spanish provenances depending on the embryogenic line

Table 4. *Somatic embryo germination and plant development in P. pinaster using mLV formulation at INRA*

Cross[a]		Line	Number of SEs			Number of plants	
♀	♂		Isolated	Germinated *(%)*	Ready for acclim. *(%)*	Acclim.[b]	Survival (4 months)
A	D	PM2	183	151 *(82)*	98 *(53)*	50	28
A	E	PM16	123	113 *(92)*	97 *(79)*	56	45
B	D	PM3	337	230 *(68)*	165 *(49)*	60	28
B	D	PM4	114	95 *(83)*	78 *(68)*	30	11
B	E	PM10	65	60 *(92)*	49 *(75)*	27	14
B	E	PM18	90	59 *(65)*	41 *(45)*	13	3
B	F	PM5	375	362 *(96)*	336 *(89)*	252	179
B	F	PM6	120	109 *(91)*	101 *(84)*	70	41
C	D	PM12	332	202 *(61)*	179 *(54)*	84	79
C	D	PM13	197	152 *(77)*	128 *(65)*	61	22
C	E	PM15	83	60 *(72)*	47 *(57)*	22	14
C	F	PM9	112	80 *(71)*	59 *(53)*	26	26
Total		**2302**		**1673** *(73)*	**1378** *(60)*	751	**490** *(65)*

[a]♀: Corsican provenances; ♂: Landes breeding population
[b]Acclimatized plants

(Humánez et al. 2012). SEs germinated in light showed signs of stress (red color of the hypocotyl) whereas those placed the first 10-14 days of germination in darkness developed an elongated hypocotyl. As a result, further handling of germinating SEs is improved (Lelu-Walter et al. 2006). Aerial parts developed after 7 weeks on germination medium.

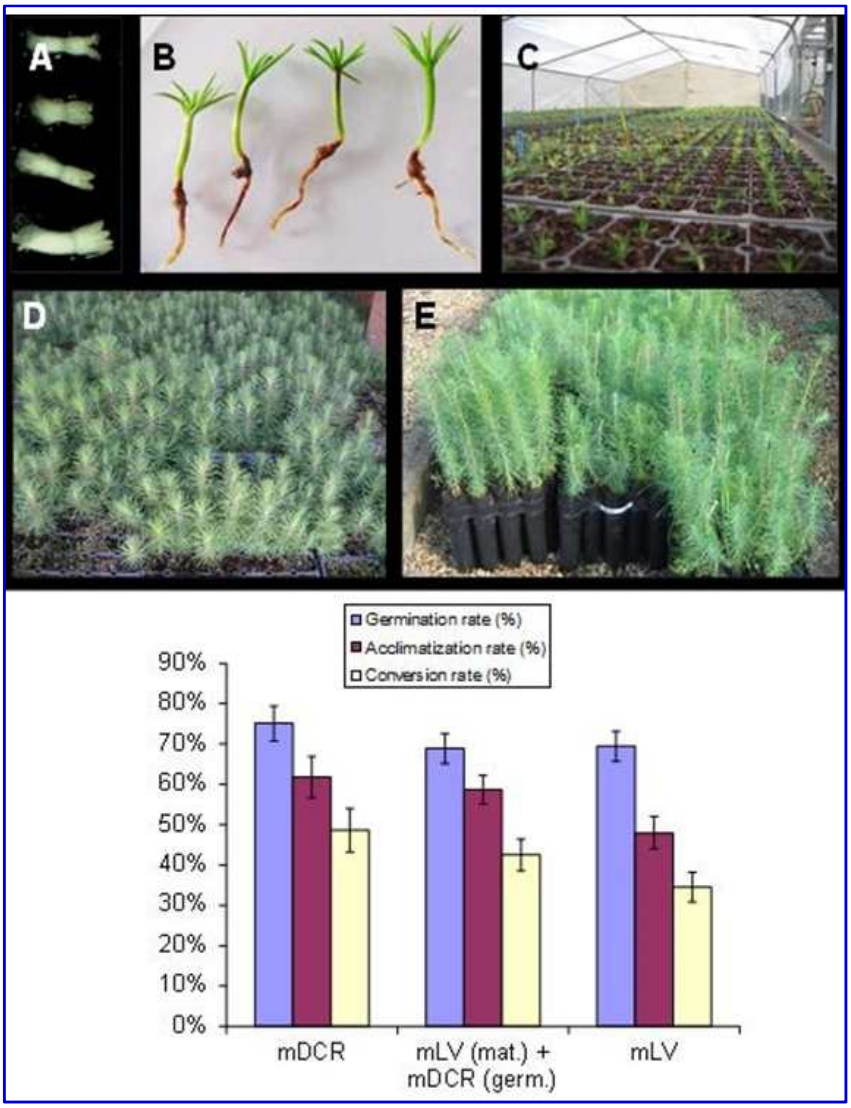

***Figure** 7. Cotyledonary SEs (**A**) germination (**B**), acclimatization (**C**) and conversion into emblings (**D, E**) and corresponding rates (%) in maritime pine as a function of basal medium used at the maturation and germination steps. Data were computed from 6 embryogenic lines originating from 5 unrelated seed families (AAF04005, AAY06006, AB774, NL04045, NL04048, PN519). mDCR: modified DCR medium from Gupta and Durzan (1985); mLV: modified LV medium from Litvay et al. (1985). Bars represent 95% confidence limits.*

Compared to mDCR, the use of Litvay-based medium formulations (mLV), at both the maturation and germination steps, resulted in a lower germination rate (69.4 vs. 75.0%), acclimatization rate (47.9 vs. 61.7%) and conversion rate (34.5 vs. 48.6%) into plantlets capable of proper growth in the greenhouse and then the nursery (Figure 7). Interestingly, when mLV formulations were used during maturation and mDCR during germination, intermediate results were obtained (Figure 7), suggesting that the negative effect of Litvay basal salts on germination rate is partially and early determined during the maturation phase.

4. Physiological and (epi)genetic status of SEs: are they similar to ZEs?

4.1 Reduced water availability induced a shift towards embryogenesis

Significant progress has been made in the development of maritime pine somatic embryogenesis but there are still technical issues that preclude full integration of this powerful vegetative propagation system into the French breeding program. As previously mentioned, maritime pine SEs require a reduction in water availability (high gellan gum concentration in the maturation medium) to reach the cotyledonary stage. This key switch, reported specifically for pine species, is not yet well understood. To facilitate the use of somatic embryogenesis for mass propagation of conifers we need a better understanding of embryo development (Jordy and Favre 2003; Tereso et al. 2007). Recently, a multi-scale, integrated analysis was used to unravel early molecular and physiological events involved in SE development (Morel et al. 2014a). Under conditions unfavourable for SE maturation (4 g L^{-1} of gellan gum in the maturation medium) both transcriptomic and proteomic profiling indicate enhanced glycolysis leading to proliferation of EMs with an increased fresh weight, which may be antagonistic to SE maturation. Under favourable conditions (9 g L^{-1} of gellan gum), we observed adaptive, ABA-mediated molecular and physiological responses to reduced water availability resulting in early transition of EMs from proliferation to the SE developmental pathway as indicated by confocal laser microscope observations, active protein synthesis, overexpression of proteins involved in cell division, embryogenesis and starch synthesis. Specific pathways (e.g. synthesis of protective secondary metabolites, regulation of oxidative stress) are also activated, apparently to overcome constraints due to culture conditions. A protein of germin type and an ubiquitine ligase appear as potential markers of early somatic embryogenesis of maritime pine, while the phosphatase protein 2C stands out as the adaptive answer to the culture environment (Morel et al. 2014a). These results may facilitate monitoring of early EM responses to maturation conditions.

4.2 When to harvest cotyledonary SE for germination?

In maritime pine, improved protocols are now available for the whole somatic embryogenesis process, i.e., from EM initiation to somatic plant regeneration (Figure 7). However, field trials established in France from somatic plant material (emblings) have consistently revealed a lower initial growth rate than the control seedlings (see below part 5). A better understanding of SE maturation is, therefore, required in order to produce high-quality, vigorous somatic plants. SEs are currently matured for 12 weeks to reach the cotyledonary stage before being germinated and converted to plantlets. Although regeneration success is highly dependent on SE quality, the harvesting date is still determined by

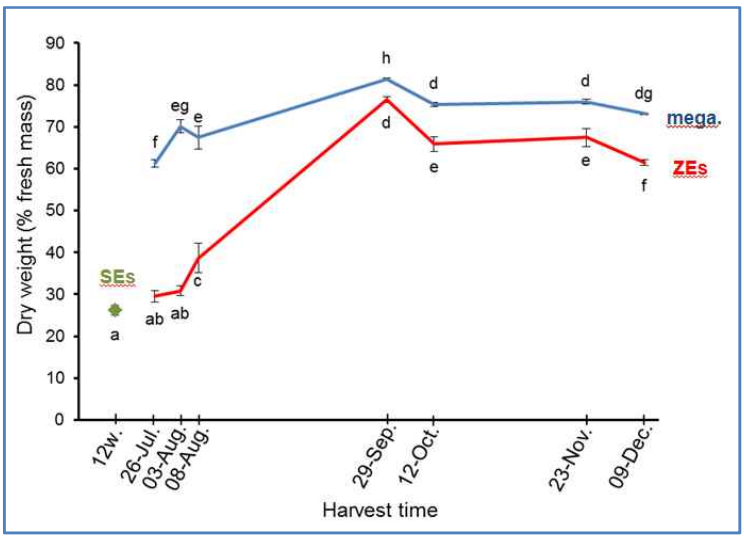

Figure 8. *Dry weight of cotyledonary embryos of somatic (SEs), zygotic origin (ZEs) or megagametophytes (mega.) as a function of harvesting time. Somatic embryos were collected after 12 weeks maturation. Zygotic embryos and megagametophytes were sampled at 7 collection dates from 26 July to 09 December 2011. Bars represent 95% confidence limits. Letters represent statistical groups defined by the Multiple Comparisons of Means method (P<0.05, N = 10).*

their morphological features. This empirical method does not provide any accurate information about embryo quality with respect to their storage compounds (proteins, carbohydrates). SEs matured for 10, 12 and 14 weeks were analyzed by carrying out biological (dry weight, water content) and biochemical measurements (total protein and carbohydrate contents). No significant difference was found between collection dates, suggesting that SE harvesting after 12 weeks of maturation is appropriate (Morel et al. 2014b). Cotyledonary SEs were then compared to various stages of cotyledonary ZEs, from fresh to fully desiccated

(from August to December). The corresponding megagametophytes were also analyzed to evaluate the impact of the maturation on this nutritive tissue. While the megagametophytes presented a slight variation in dry weight (Figure 8), and carbohydrate content (Figure 9) in comparison to that of the ZEs, their respective protein content changed about the same, but in opposite direction, highlighting the transfer of proteins from the nutritive tissue to the embryos (Figure 10). The similarity of the 12-week-old SEs with the fresh cotyledonary ZEs sampled from late July to early August (Figures 8, 9, 10) was confirmed by a hierarchical ascendant cluster analysis with 9 variables. Both types of embryo exhibited similar carbohydrate and protein content and signatures.

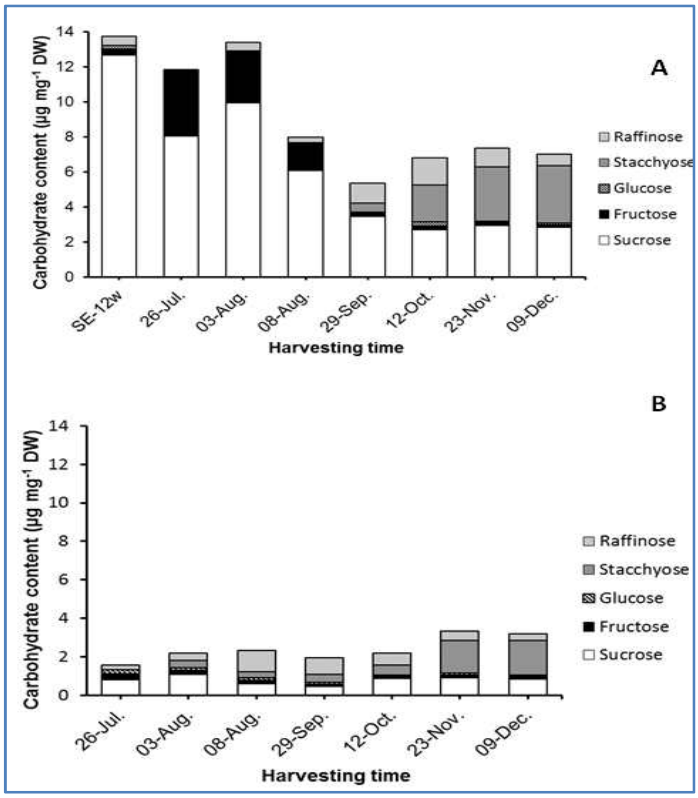

Figure 9. *Changes in carbohydrate content in (**A**) cotyledonary embryos of somatic (SEs) or zygotic origin (ZEs) and in (**B**) megagametophytes as a function of harvesting time. SEs were collected after 12 weeks maturation (SE-12w). ZEs and megagametophytes were sampled at 7 collection dates from 26 July to 09 December, 2011. Bars represent 95% confidence limits.*

This high level of similarity was evaluated at 94.5% according to a proteome profiling test. Highly expressed proteins included storage, stress-related, late embryogenesis abundant (LEA) and energy metabolism proteins. By

comparing overexpressed proteins in developing and cotyledonary SEs or ZEs, some (23 proteins) could be identified as candidate biomarkers for the late, cotyledonary stage. Of these, 18 belonged to five large families of proteins including five HSPs, four LEAs and two other stress-related proteins (aldose reductase, 6-phosphogluconate dehydrogenase), five storage proteins and two proteins involved in purine metabolism (adenosine kinase 2, SAM synthase; Morel et al. 2014b). Results also suggest that improvements of SEs quality may be achieved if the current maturation conditions are refined.

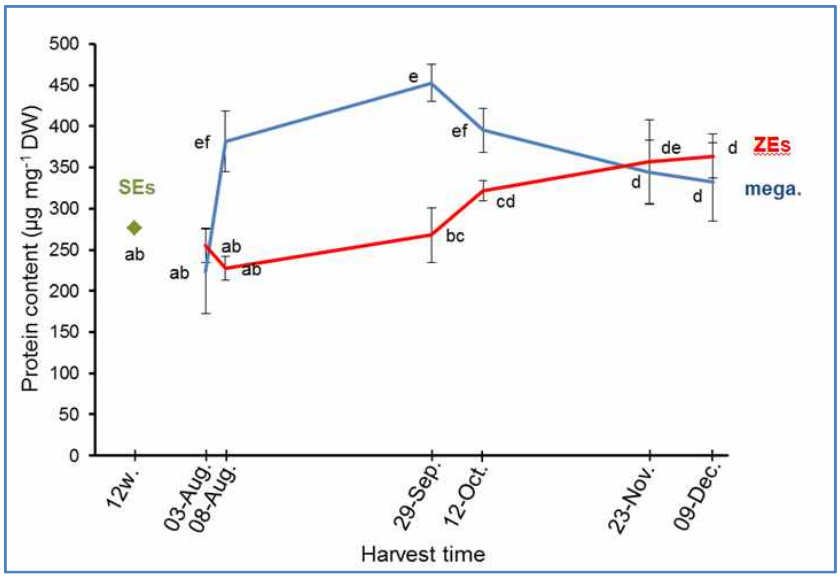

Figure 10. *Quantitative analysis of total proteins in cotyledonary somatic embryo (SEs) matured for 12 weeks, compared to cotyledonary zygotic embryos (ZEs) and their respective megagametophytes (mega.) during maturation. Bars represent 95% confidence limits. Letters represent statistical groups defined by the Multiple Comparisons of Means method (P<0.05, N = 5).*

5. Field testing of somatic seedlings

Data collected from field trials are crucial information to validate the somatic embryogenesis technology, i.e., it is necessary to compare growth and phenology traits of emblings with those of control seedlings. A total of 8 field trials have been established since 1999 by FCBA (7) and INRA (1) with a total of about 3200 somatic plants in test. Each trial is comparing 11 to 78 lines originating from 6 to 12 elite families. First trials were performed in the nursery whereas more recent trials (since 2004) are established in forest conditions. Most plants were initially produced from lines cultivated with DCR-based media, especially at FCBA, but in recent field trials most plants were obtained from lines cultivated

with Litvay-based media. A mean of 91% survival of emblings occurred at age 2 in these field trials. The older trial was planted at a FCBA nursery plot in 1999 (59 plants from 12 clones) and the trees are now entering their adult reproductive phase with regular cone production (Figure 11). Male flowering was classically observed as early as at age 5. Following the last measurement at age 12, somatic trees were 6.5-12.1 m in height with diameters in the range 9-29 cm. These measurements are similar to those of control seedlings at the same plot which were 9.6-11.2 m in height and 19-33 cm in diameter. This trial demonstrates that somatic seedlings could complete the juvenile and adult vegetative growth phases.

Figure 11. *Maritime pine emblings planted by FCBA in 1999 at a nursery plot (Sivaillan, France). The 12-year-old trees are entering their adult reproductive phase (cone production can be observed).*

In another trial planted by FCBA in 2004 (99 plants from 35 lines obtained from 7 families), somatic plants are developing into trees with normal phenotypic behaviour (Figure 12). Mean height at age 7 years (5.71 m) is however significantly lower than that of control seedlings (7.03 m) but no significant difference could be detected for the mean relative increase in height since planting. We conclude that somatic trees developed at a lower initial growth rate than control seedlings. Such a low initial growth was similarly observed in a field trial of 24 somatic clones planted in 2004-2005 (Figure 13). At age 6 years, the height of some clones was found to be similar to that of seedling controls (e.g. clones 25C, 29C, DE737, CM815, ET816). Computing the mean relative increase in height since planting revealed that some clones performed as well as control seedlings and even better in some cases (e.g., clones 29C, PN6128, NM626, NM18c). These results are very encouraging and suggest that initial low growth rate of somatic clones can be overcome after a few years and can be compensated for by selecting within each family the top elite clones. Although full genetic gains cannot be obtained currently with somatic seedlings because of their low initial growth it is expected from field trials that opportunities will exist for selection of clones with improved adaptability and performance to sustain forest productivity in maritime pine plantation forestry (see Wahid et al. 2012).

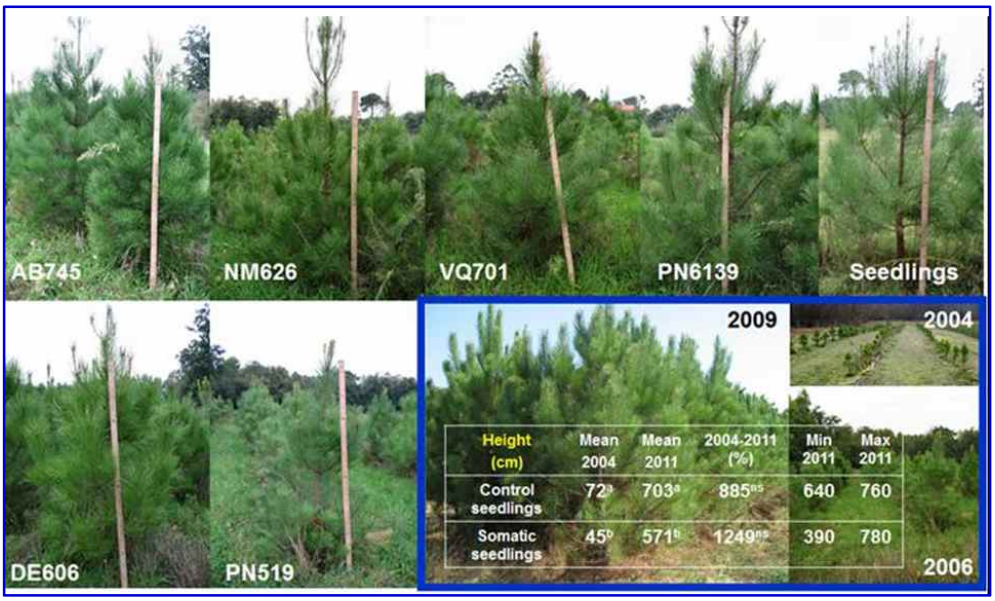

Figure 12. *Somatic clones obtained from 6 embryogenic lines and control seedlings 2.5 years after plantation at a nursery plot (2004, Sivaillan, France). Blue box: a general view of the field trial in 2004, 2006 and 2009. The Table is giving mean height (2004, 2011), mean relative increase between 2004 and 2011 (%), minimal (Min) and maximal (Max) height (2011) for somatic and control seedlings. Significant differences between means are indicated by different letters. ns: non-significant (p< 0.05).*

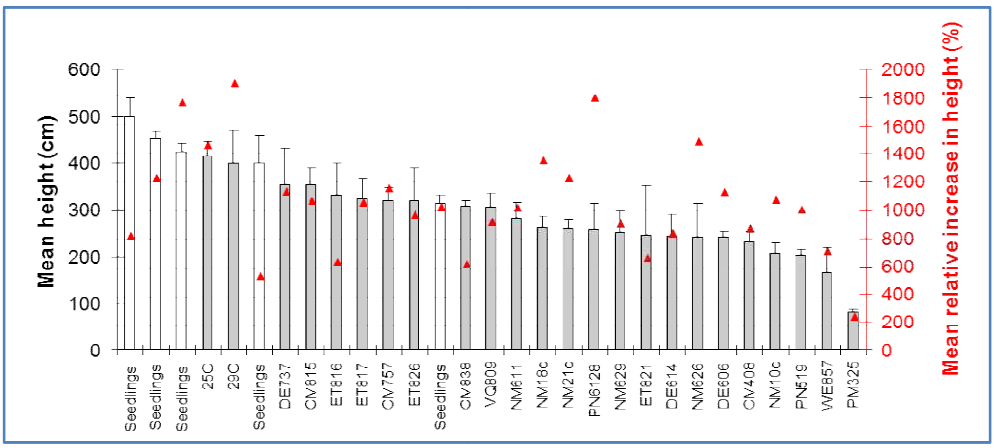

***Figure 13**. Mean height and relative increase in height at age 6 of 24 FCBA or INRA somatic clones (in grey) and 5 control seedlings lots (in white) planted by FCBA in a forest field trial in 2004-2005 (Landriole, France). Control seedlings are either related with clones (genetic controls) or with VF1 varieties (improved controls). Bars represent 95% confidence limits for mean height.*

6. Challenges with somatic embryogenesis in maritime pine

6.1 Aging in embryogenic cultures

Cryopreservation of plant material is not only useful but necessary due to the instability of the physiological and/or DNA methylation state of most embryogenic lines of maritime pine. Regarding short- to mid-term propagation goals, this could easily be compensated for by selecting a few stable lines. In case this instability problem could be solved, or at least significantly reduced, cryopreservation would still be required for securing the long-term availability of lines, particularly for still fragile, freshly initiated ones, and also for line maintenance at low cost when their continuous propagation is not required. In breeding operations, cryopreservation would still be required during the testing of emblings in field plots until the age of final confirmation of their individual clone performance and of their ability to adapt to environmental parameters, i.e., water availability, soil fertility and pest occurrence. Nevertheless, we can anticipate that in the future massive propagation of new clones could directly be launched shortly after initiation from seeds on the basis of marker-assisted or genomic selection. Early phenotype selection among individual immature ZEs may become possible, thus bypassing the need for lengthy initial testing.

The instability of lines has often been described for many plants in terms of "aging" or performance decline (Bhaskaran and Smith 1990). This was described early in the history of conifer somatic embryogenesis in the case of spruce. Degenerating lines, showing an altered microscopic and/or macroscopic

morphology, typically showed a decreased maturation ability. In some cases, this phenomenon was related, or proposed to be related, to the occurrence of mutation in particular genes involved in key steps of embryo morphogenesis (Fourré et al. 1997; Egertsdotter and Von Arnold 1995). Further insights into somatic embryogenesis of model plants and conifers as well (Rose and Nolan 2006; Smertenko and Bozhkov 2014; Zhu et al. 2014; Zhu 2015), pointed out the genetic control of the process. However detailed studies of allelic variability in these genes are still lacking. Regarding maritime pine, Breton et al. (2005, 2006) showed that culture medium as well as various other cultivation parameters like subculture frequency can delay this evolution. It is noteworthy that the same full-sib families could give rise to lines with contrasting behaviour, particularly in terms of sensibility to factors impacting their morphology and associated maturation ability.

6.2 Variability in culture

Tissue culture media vary from lab to lab or even among operators or batches because some components and/or preparation steps are not disclosed or fully described. Different batches of the same medium prepared by different operators could substantially differ in their ability to sustain a proper evolution or reaction of the cultured plant material. Ramarosandratana et al. (2001b) described how establishing cell lines from different EMs can lead to cell populations with different SE maturation ability. Furthermore Breton et al. (2006) reported that variation in maturation yield in maritime pine is independent from spatial factors (the dish) and temporal ones (different sub-lines from the same cryopreserved stock). Spatial heterogeneity probably results in a batch-to-batch or container-to-container variability of maturation yield. Suboptimal conditions can impact the overall cost of the plant production process.

Genetic variation has been shown to represent a major source of variability in somatic embryogenesis of various plant species including conifers (Wareing and Phillips 1970; Henry et al.1994; Nestares et al. 2002; MacKay et al. 2006; Park et al. 1994, 2006; Pinto et al. 2008). This could be seen as a case of the common phenomenon of genetic (relative) recalcitrance to vegetative propagation as described by McCown (2000), Bonga et al. (2010), Elhiti and Stasolla (2012), among many others. Recent results point out the modulation of recalcitrance by both genetic and epigenetic factors (reviewed by Mahdavi-Darvari et al. 2015).

Ramarosandratana et al. (2001a) describe the variable sensitivity among maritime pine lines to three components of the maturation medium (i.e., osmoticum, sugar and gelling agents). Similarly, we showed at FCBA (unpublished) through testing of a few hundred lines the vast initial variability of the response of full-sib lines to alternative maturation protocols.

6.3 Towards industrial application of somatic embryogenesis

The use of bioreactors or other liquid medium-based *in vitro* culture containers has long been proposed as an efficient solution for the production of high quality mature conifer SEs (Attree et al.1994; Tautorus et 1994; Moorhouse et. 1996; Timmis et al. 1998; Gupta and Timmis 2005) but there is no published case of real commercial application. Several companies have claimed some success recently, e.g., Arborgen (Becwar et al 2012), Sweetree (Egertsdotter and Johnson 2014) and Weyerheauser (Swanda 2014).

The biggest challenge toward industrial scale-up of conifer somatic embryogenesis is the genetic as well as physical and temporal heterogeneity of the plant material in response to the treatments aimed at obtaining high quality forest plants. This heterogeneity results in a high variability of results and unpredictability of operational scale production. This problem is less pronounced for some species, like hybrid larch and Norway spruce (Lelu-Walter et al. 2013; Thompson 2014), than for maritime pine.

For maritime pine, the current challenge is the quality of the plant material resulting from the conversion of cotyledonary SEs into somatic seedlings. Indeed, while we produced a huge number of mature embryos over the years at FCBA (nearly half a million) and raised *ex vitro* more than fifty thousand plants, and established several field tests (> 3200 plants, see part 5), the results were unsatisfactory because the initial growth of emblings was slower than that of seedlings. However, as for spruce species (Grossnickle and Major 1994a, b), recovery from slow growth occurred over time.

7. Conclusions: prospects for industrial application

We have described the state and challenges facing the industrial deployment of somatic embryogenesis of forest trees in general and with some details regarding maritime pine (Lelu-Walter et al. 2013). This subject was also dealt with by Thompson (2014), who described the prospects for solving problems such as social acceptance and hesitance of nursery managers and foresters to use SE in their operations.

For maritime pine, most of the technical steps (i.e., initiation, multiplication and cryopreservation) are working sufficiently well with a high rate of capturing genotypes at the initiation step (65-85%). However, subsequent steps towards the regeneration of plants that meet the quality standard required for commercial purposes need further investigation. Low genotype capture at the maturation step (< 50%, see part 3) as well as inadequate embryo quality compared to the quality of fresh cotyledonary ZEs (see part 4) are both currently precluding the regular production, at acceptable cost, of enough plants from a suitable number

of clones. It is likely – but this needs to be validated - that several aspects of these difficulties are interrelated and that improvement at any step will impact positively the yield at other steps. Expected future improvements probably will also help to obtain plant material that is suitable for automatized processing which could result in decreased production cost.

The production of healthy vigorous plants able to sustain harsh weed competition on low fertility sites is a prerequisite for acceptance of SE plant material by breeders, nursery operators and foresters. To achieve such improvements would require close collaboration between academic and private teams. For maritime pine such a collaboration has been in effect since 2004 between INRA and FCBA and has already resulted in significant achievements. Improved knowledge of the somatic embryogenesis process and embryo development, particularly of its molecular aspects (de Vega-Bartol et al. 2013; Morel et al. 2014a,b, Trontin et al. 2015), are thought to provide new opportunities for further refinement of somatic embryogenesis in maritime pine.

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9. Authors contribution

JFT carried out somatic embryogenesis experiments, conceived the design of the study and drafted the manuscript. CT conceived the design of the study, carried out protein analysis and drafted the manuscript. AM carried out protein analysis and helped to draft the manuscript. LH conceived the design of the study and drafted the manuscript. MALW carried out somatic embryogenesis

experiments, conceived the design of the study and its coordination, and drafted the manuscript. All authors red and approved the final manuscript.

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