Chapter 19
Somatic Embryogenesis for More Effective Breeding and Deployment of Improved Varieties in *Pinus* spp.: Bottlenecks and Recent Advances

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Abstract Global transition towards a bioeconomy sets new demands for wood supply (bioenergy, biomaterials, biochemicals, etc.), and the forestry sector is also expected to help mitigate climate change by increasing carbon fixation. For increased biomass production, the use of improved, genetically superior materials becomes a necessity, and vegetative propagation of elite genotypes provides a potential delivery mechanism for this. Vegetative propagation through somatic embryogenesis alone or in combination with rooted cuttings obtained from somatic young trees can facilitate both tree breeding (greater selection accuracy and gains,

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breeding archives of donor material for making crosses after selection) and the implementation of deployment strategies for improved reforestation materials. To achieve these goals, progress in the efficiency of pine somatic embryogenesis biotechnology has been made for a few commercial pine species, and a better understanding has been gained of the molecular mechanisms underpinning somatic and zygotic embryo development.

19.1 Introduction

Pines (*Pinus* spp.) are native to most of the Northern Hemisphere and are also extensively planted in many parts of the Southern Hemisphere, with 119 species in the genus (Tang and Newton 2008). *Pinus* spp. make up 46 % of the estimated 53.4 million ha of planted production forest worldwide, with *Eucalyptus* spp. being the next largest at 26 % (FSC Strategic Review on the Future of Forest Plantations 2012). The most planted conifer in the world, originally from California (USA), is Monterey pine (*Pinus radiata*) covering over 10 million acres (4.05 million ha) of plantations. Other species include loblolly pine (*Pinus taeda*), a dominant commercially planted species in Southeastern USA, where it covers 25 million acres (10.1 million ha); Scots pine (*P. sylvestris*), a European species, is also planted in North America as well as in New Zealand (Tang and Newton 2008), and maritime pine (*P. pinaster*), native to the Mediterranean basin (ca. 4 million ha), is the most important timber species cultivated in France, Spain and Portugal, where it is the dominant species in over 2.3 million ha. Breeding programmes have been established for these commercially important pine species and protocols for somatic embryogenesis (SE) are being refined as a means of vegetative propagation (Klimaszewska et al. 2007, 2016). Conifer SE is important for tree improvement owing to its potential for making possible the selection and mass propagation of elite genotypes from a broad genetic base. In particular, it offers significant opportunities to improve management of breeding populations and to accelerate delivery of improved material to plantations through clonal forestry (Park et al. 1998; Cyr and Klimaszewska 2002; also see below *Implementation of SE in tree breeding and forest regeneration*).

A typical explant for initiation of SE in pines (and other conifers) is an immature zygotic embryo (*ze*), most frequently at the early to late cleavage polyembryony through early to late dominance stages (von Aderkas et al. 1991; Cairney and Pullman 2007), with a few species best responding at the cotyledonary stage of development. Since the first reports on SE induction in *P. lambertiana* and *P. taeda* (Gupta and Durzan 1986, 1987), a large number of publications reporting the production of somatic trees through SE have been published, the majority of which concerns the most economically important timber species such as *P. taeda* (Pullman and Bucalo 2014 and references therein), *P. radiata* (Hargreaves et al. 2011), *P. pinaster* (Lelu-Walter et al. 2006; Trontin et al. 2016a) and *P. sylvestris* (Lelu-Walter et al. 2008; Aronen 2016). Because SE in pines and other conifers is a

multistage process, each stage requires adjustments of in vitro treatments to maximize its efficiency. After SE is initiated and proliferating embryonal masses (EM) reach several hundreds of mg in fresh mass, they have to be bulked up through serial subcultures, most frequently on media of the same composition, until the required amount of EM is obtained. At this time, a suitable amount of EM is cryopreserved and stored in liquid nitrogen to ensure ample supply of EM lines (genotypes) for future tree production, when the progenies (regenerated trees from EM lines) have been field proven. After storage, the next crucial stage in conifer SE is the development and maturation of EM into mature somatic embryos (*se*) on a medium of different composition. Once these are mature, which is usually judged by visual inspection, they may be germinated on a different medium until plantlets are obtained. The next step is to transfer somatic seedlings (*ss*) to a soil-less substrate for acclimatization in a greenhouse before their transfer to a nursery for further growth and quality assessment before plantation in the field. Notwithstanding the recent progress made with respect to the optimization of laboratory protocols for SE of pine species, there are still some problems that need to be solved. Depending on the species, these problems include either all or some of the following: low SE initiation frequency from the seed explants, reduction or cessation of *se* regeneration capacity concomitant with the increased chronological age of EM; low genotype capture at the maturation step and low number of mature *se*; poor *se* quality at the end of the maturation stage; and inferior initial growth of *ss* compared with seedlings in the field (Fig. 19.1). In this review, we highlight the progress made but also the bottlenecks in pine SE, the ongoing research aimed at understanding the underlying causes, and we recommend potential solutions.

19.2 Induction of Somatic Embryogenesis in *Pinus* spp.

19.2.1 Initiation from Seed Embryos

In *Pinus* spp., SE is initiated most efficiently from immature *ze* enclosed within the whole megagametophyte. However, in some species such as *P. radiata*, SE induction has also been induced successfully using excised *ze* (Table 19.1). The two most commonly used media for pine SE are DCR (Gupta and Durzan 1985) and LV (Litvay et al. 1985) as modified by Klimaszewska et al. (2000) (mLV).

mLV has been initially used for SE in *P. strobus* with a high degree of success with multiple seed families (Klimaszewska et al. 2001). Later, it has been utilized for SE of *P. strobus* x *P. wallichiana* F2 hybrid seeds with initiation frequencies of over 50 % in control-pollinated seed families (Daoust et al. 2009). Cultures on mLV consistently produce high initiation rates in maritime pine (Table 19.1) with both French (Lelu-Walter et al. 2006; Trontin et al. 2016a) and Spanish (Humánez et al. 2012) seed families. In maritime pine, comparison of DCR (Gupta and Durzan 1985) and modified DCR (Breton et al. 2005) (mDCR) in a parallel experiment revealed that SE initiation rates were reduced by approximately 50 % compared

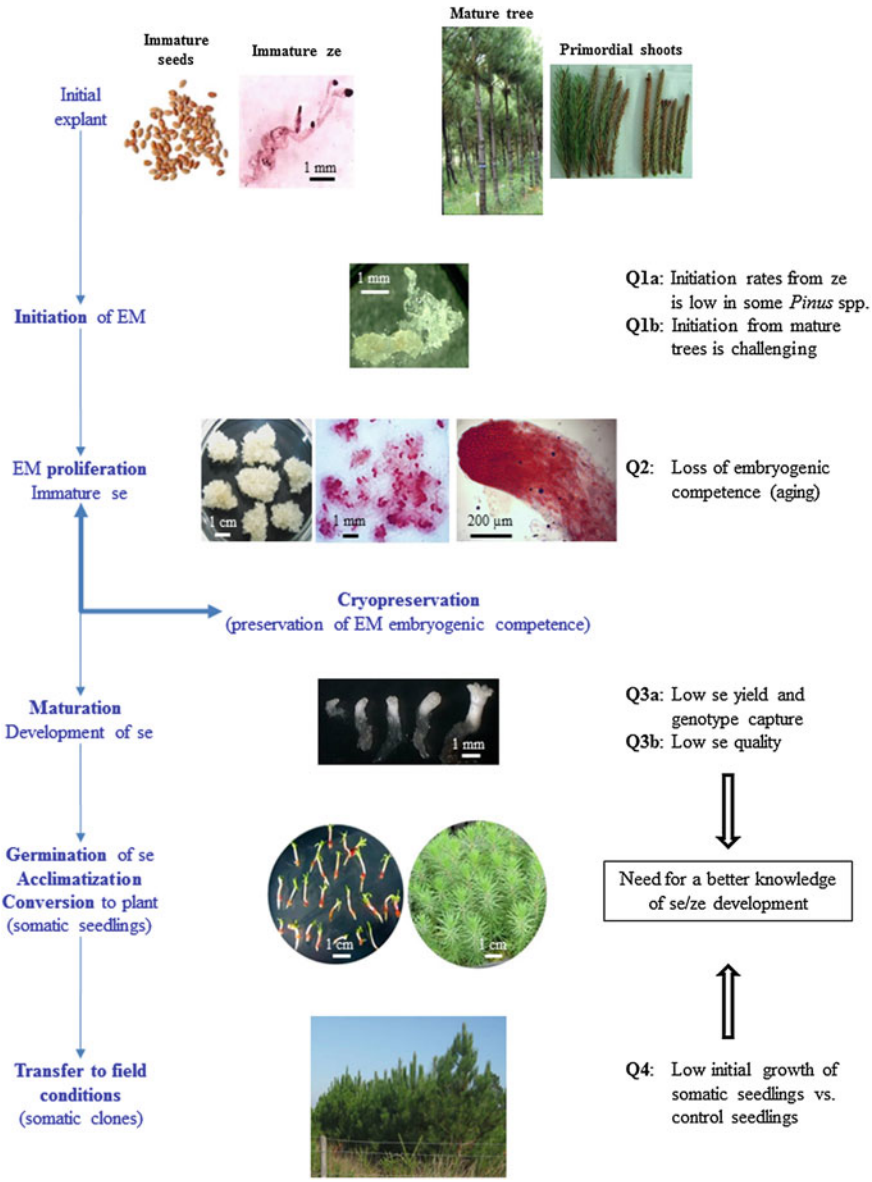

Fig. 19.1 Stages of somatic embryogenesis in pines and critical questions (Q). Illustrations are from maritime pine (FCBA/INRA). *EM* embryonal mass; *se* somatic embryo; *ze* zygotic embryo

with mLV. The data, based on a large selection of full- or half-sib seed families, showed that the mean initiation rate was 67.5 % on mLV and only 23.3 % on mDCR (Trontin et al. 2016a). Plant growth regulators, usually an auxin (2,4-D/2,4-dichlorophenoxyacetic acid) and a cytokinin (BA/benzyladenine), are

Table 19.1 Initiation rate of somatic embryogenesis in *Pinus* spp.: some recent reports

Pinus species	*Ze* dev. stage	Seed origin	Initiation response (% range)	Reference
radiata	*Dissected ze*			
	Pre-coty.	19 open PT	47–97	Hargreaves et al. (2009)
	Pre-coty.	20 control PT	44–93	Hargreaves et al. (2011)
	Undissected ze			
densiflora	n/a	12 open PT	n/a	Kim and Moon (2014)
halepensis	Pre-coty.	7 open PT	1–7	Montalbán et al. (2013)
luchuensis	Coty.	1 open PT	1	Hosoi and Maruyama (2012)
nigra	n/a	12 open PT	1–10	Salaj et al. (2014)
oocarpa	Early stage	2 open PT	2–9	Lara-Chavez et al. (2011)
pinaster	Dominant	5 open PT	0–82	Humánez et al. (2012)
	Dominant	16 control, 4 open PT	65–96	Trontin et al. (2016a)
pinea	Pre-coty.	5 open PT	<1	Carneros et al. (2009)
radiata	Pre-coty.	20 control PT	0–73	Hargreaves et al. (2011)
		2 open PT	24–60	Montalbán et al. (2015)
sylvestris	Early stage	6 open PT	0–30	Aronen et al. (2009)
strobus x *wallichiana*, F2	Dominant	12 control PT	52	Daoust et al. (2009)
taeda	Early to Pre-coty.	11 control, 1 open PT	6–43	Pullman et al. (2015)

Coty. cotyledonary; *dev. stage* development stage; *ze* zygotic embryo; *PT* pollinated trees; *n/a* not available

required for SE initiation at a high rate on mLV (Lelu-Walter et al. 2006; Humánez et al. 2012), but initiation can also be achieved at a relatively high frequency without PGR in *P. pinaster* (Lelu et al. 1999). Alternatively, in mLV (but not mDCR), 2,4-D and BA could be substituted by a potent cytokinin (the phenylurea CPPU), which increased SE induction efficiency to 77 % compared with 34 % in its absence (Park et al. 2006). Results from multiyear experiments conducted with maritime pine at FCBA established the optimal CPPU concentration at 1 µM (Trontin et al. 2016a).

As it is a potent cytokinin with a putative role in ageing and vigour in pine (Valdés et al. 2003; Klimaszewska et al. 2009), CPPU supplementation during the

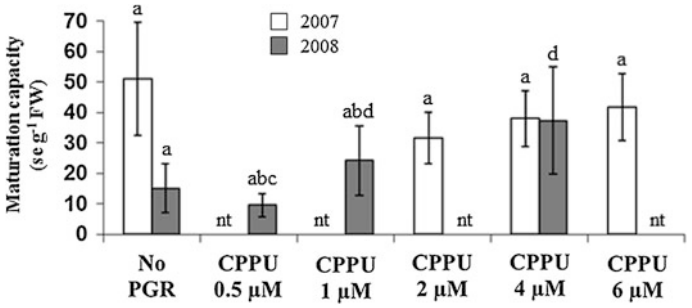

Fig. 19.2 Mean maturation capacity of maritime pine embryogenic lines initiated at the FCBA in 2007 or 2008 on mLV medium containing various concentrations of phenylurea CPPU (0–6 µM). 2007: 19–20 lines (from four families) matured per condition after 16–29 weeks of subculture (on average, post-initiation) on multiplication medium (nine maturation experiments with 0.5 g FW/line). 2008: 14–19 lines (from four families) matured per condition after 12–22 weeks of subculture (on average, post-initiation) on multiplication medium (nine maturation experiments with 1 g FW/line). mLV medium (modified LV medium from Litvay et al. 1985) was used for both multiplication and maturation of embryogenic lines. *FW* fresh weight; nt: not tested; *se*: somatic embryo. *Bars* represent 95 % confidence limits. For each year, significant variations between means (t-tests, $\alpha = 0.05$) are indicated by different letters

induction phase could have an adverse and delayed effect on the maturation capacity of initiated embryogenic lines. Hence, experiments with maritime pine were carried out in 2007 and in 2008 with 14–20 EM lines (from four seed families) to compare *se* maturation yields after 12–29 weeks of subculture following induction (Fig. 19.2). Mean maturation capacity of lines initiated on media with CPPU at 0.5–6 µM was compared with lines initiated without CPPU (no PGR). In 2007, no CPPU effect (2–6 µM) could be detected. In 2008, CPPU (0.5–4 µM) was confirmed as a good substitute for 2,4-D/BA in initiation medium as maturation capacity was found to be either similar (0.5–1 µM CPPU) or even significantly higher (4 µM CPPU) compared with control lines initiated without PGR. Interestingly, a general trend for increased maturation capacity as a function of CPPU concentration was observed during these experiments with some significant differences in 2008, i.e. higher *se* yield for lines initiated with 1–4 µM compared with 0.5 µM CPPU (Fig. 19.2). These results suggest that the type and concentration of PGR used in the SE induction medium may affect other SE steps up to maturation step (*se* development).

In these multiyear experiments, the seed family effect remained significant, but the genotype capture was in the range of 65–96 % and no adverse effect of CPPU on the development of *se* during maturation was observed. Hence, SE induction frequency in maritime pine fulfils the requirements for implementation of this biotechnology in the French breeding programme (Trontin et al. 2016a).

In *P. radiata*, another modified LV medium (GLITZ) was also found to be very effective for SE initiation with special benefits for immature excised *ze* (Hargreaves et al. 2009, 2011). Results with 19 open- and 20 control-pollinated seed families have shown initiation rates in excess of 50 % irrespective of the collection time of

immature cones; at the optimum *ze* developmental stage for each cross, an average of 70 % of the explants produced established cell lines. Similar to *P. pinaster*, these are the two *Pinus* spp. that can no longer be considered recalcitrant to SE in reference to the initiation stage (Table 19.1).

19.2.2 Initiation from Mature Trees

SE initiation from vegetative tissues of individual trees at adult vegetative or reproductive growth phases, when the tree's characteristics and growth performance are demonstrated, is highly desirable for implementation of efficient multi-varietal forestry. This has never been more important than today with the increasing pressures of a changing climate and new disease incursions into our global forestry environment. The direct propagation of selected trees would significantly reduce the costs and efforts of delivering elite varieties in commercial plantation forestry. In maritime pine, SE from mature trees could produce a new variety ready for deployment in less than 5 years (Fig. 19.3). In contrast, SE from seed embryos of unknown performance requires that somatic clonal trees be field tested until they reach the reproductive growth phase (10–15 years). Then, the selected tree varieties may be mass propagated from the cryopreserved juvenile stock established at the

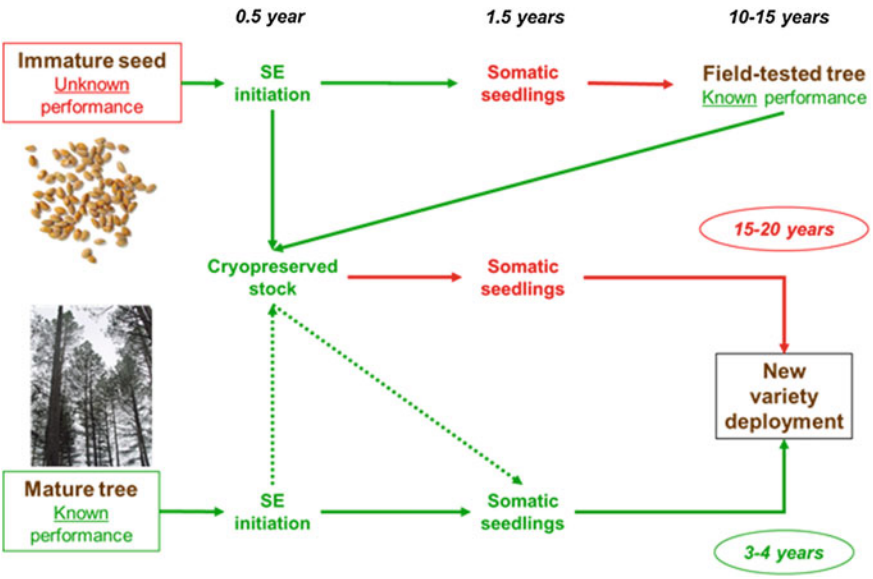

Fig. 19.3 Somatic embryogenesis from immature seed or mature tree in maritime pine as a means to implement multi-varietal forestry through the deployment of tested/selected elite varieties. *Dashed lines* indicated optional steps

time of SE initiation. Therefore, the whole process for new variety development takes over 15–20 years to be completed (Fig. 19.3).

Unfortunately, SE initiation from mature tree tissues is still as challenging in pines as in other conifers. In the recent international, multiyear project conducted with six pine species (*P. contorta*, *P. patula*, *P. pinaster*, *P. radiata*, *P. strobus* and *P. sylvestris*) based on published and original protocols (reviewed in Trontin et al. 2016c), strong evidence for SE initiation from primordial shoot explants was reported for only one species (*P. sylvestris*, Aronen and Ryynänen, see Trontin et al. 2016c). A few abnormal or cotyledonary *se* were obtained in two embryogenic lines expressing embryogenesis-related genes (*VP1*, *WOX2*), but these *se* were developmentally arrested and did not germinate. Moreover, embryogenic lines were apparently genetically unstable as various mutations were detected at several microsatellite loci. In other species, a few initiated cell aggregates were shown to have embryogenic-like micromorphology (*P. pinaster*, Trontin et al. 2016c) or to express putative embryogenesis-related genes (reviewed in Trontin et al. 2016b; Miguel et al. 2016), such as *LEC1* in *P. radiata* (Garcia-Mendiguren et al. 2015) or *WOX2* in *P. contorta* (Park et al. 2010). However, those cell aggregates could not be sustainably propagated. Overall, these results suggest that some initial stages of SE induction did occur in primordial shoot tissues of mature pine trees, but the process did not progress. In conifers, demonstrated evidence for SE initiation from mature trees, i.e. with plant regeneration, has only been reported from somatic trees in *Picea abies* (Harvengt et al. 2001) and *Picea glauca* (reviewed in Klimaszewska and Rutledge 2016). Apparently, *se*-derived trees may have higher embryogenic potential than seed-derived trees (Klimaszewska et al. 2011; Trontin et al. unpublished).

19.3 Multiplication of EM and Progressive Reduction in Somatic Embryo Production Capacity as a Result of the Culture's Increased Chronological Age

Rapid EM multiplication is easily achieved, especially when using the culture method over a filter paper disk as first developed for *P. strobus* (Klimaszewska and Smith 1997). EM culture subdivision during proliferation at both spatial (Petri dish) and temporal levels (subline) did not affect the *se* production capacity of *P. pinaster* EM lines (Breton et al. 2006), indicating that environmental conditions were homogeneous and standardized. In contrast, EM growth was strongly impacted by cell density on the filter paper (optimal in the range of 50–100 mg fresh mass for most embryogenic lines) and medium formulation. mLV promoted a higher proliferation rate (up to 1500 % relative increase in fresh mass within 2 weeks) compared with mDCR (Trontin et al. 2016a).

Cryopreservation of embryogenic lines shortly after initiation (usually within 2–4 months post-initiation) is necessary because *se* production capacity decreases

rapidly during proliferation (between 6–10 months of subculture; Breton et al. 2006; Trontin et al. 2011). Considering the ratio of fully developed cotyledonary *se* to precotyledonary and abnormal *se* at various sampling times during proliferation, Breton et al. (2006) showed that the maturation of *se* was qualitatively unchanged, but that it quantitatively and progressively reduced as the number of consecutive subcultures and, hence, the chronological age of the cultures increased.

Ageing of EM cultures during proliferation not only impacts yield; it also impacts cotyledonary *se* quality. Breton et al. (2006) observed a significant reduction in *se* total mean length from ca. 3 mm in young sublines (subcultured for 10–12 weeks) to less than 2 mm in older sublines (subcultured for >30 weeks). In particular, an overall size reduction of about 33 % in the hypocotyl region occurred after 15 weeks of subculturing (Fig. 19.4). As expected, the smaller size of the cotyledonary *se* resulted in a lower embryo quality that showed lower germination rates compared with se matured from younger cultures. In an experiment with six embryogenic lines subcultured for 5–22 weeks, a clear trend towards decreased germination was observed, particularly after 12 weeks at which time se germination dropped below 50 % (Fig. 19.5). However, sporadic lines (genotypes) produced *se* that germinated at a relatively high frequency even after more than 20 subcultures.

The ageing mechanisms responsible for the progressive loss of *se* productivity in embryogenic lines during prolonged EM proliferation are still not well understood in pines as in other conifer genera. Variation of both genetic and/or episomaclonal origin can be involved in the instability of in vitro propagated embryogenic lines (reviewed by Miguel et al. 2016; also, see below *Molecular aspects of SE and ZE in pines*).

Interestingly, in maritime pine, the ageing process could be delayed (but not suppressed) by more frequent subcultures on fresh proliferation medium with

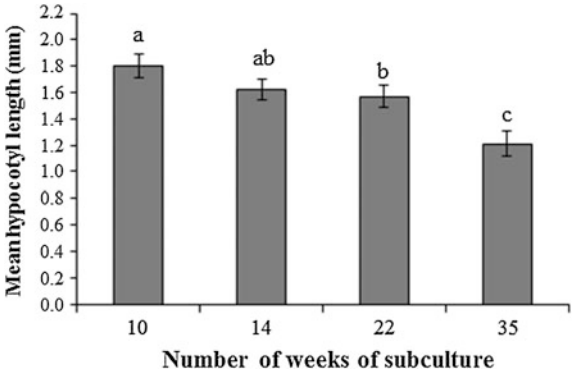

Fig. 19.4 Mean hypocotyl length of cotyledonary somatic embryos (*se*) in maritime pine (PN519 embryogenic line) as a function of the number of weeks of proliferation since EM reactivation from the cryopreserved stock. PN519 was proliferated and matured at the FCBA on mLV medium (modified LV from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. *Bars* represent 95 % confidence limits. For each sampling date (N = 36–90 cotyledonary *se*), significant variation between means (t-tests, $\alpha = 0.05$) is indicated by different letters

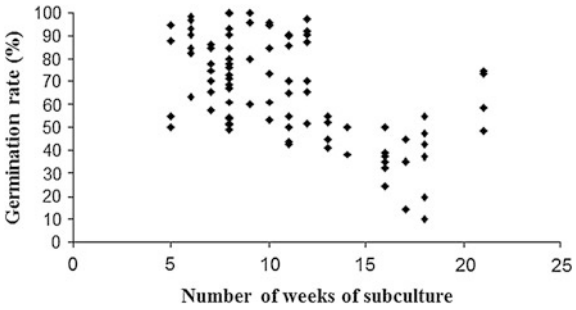

Fig. 19.5 Germination capacity of cotyledonary somatic embryos (*se*) in maritime pine as a function of the number of weeks of subculture on proliferation medium since EM reactivation from the cryopreserved stock. Data have been collected at the FCBA from six lines originating from five families (PN519, AB774, AAF04005, AAY06006, NL04045, NL04048). Lines were proliferated and matured on mLV medium (modified LV from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. ANOVA of this dataset (three SE classes: 5–8, 9–12 or >12 weeks of subculture) revealed a significant impact of subculture duration ($F = 21.01$, $p < 0.01$) and genotype ($F = 13.46$, $p < 0.01$) on germination rates and also a significant interaction between genotype and subculture duration ($F = 2.12$, $p < 0.05$)

maltose instead of sucrose and no PGR (Breton et al. 2005). For the time being, cryopreservation is still necessary to retain young clones of initiated lines until alternative culture conditions are discovered to overcome the ageing problem. Alternatively, initiation of secondary SE from cotyledonary *se* can be used to "rejuvenate" aged embryogenic lines. It has been shown that secondary SE lines initiated from three out of four tested genotypes of maritime pine had similar or improved *se* maturation capacity compared with young lines initiated from *ze* (Klimaszewska et al. 2009). Various genes may be involved in the "rejuvenation" process. For example, Uddenberg et al. (2011) reported that initiation rate of secondary embryogenesis from young germinants in Scots pine was significantly improved by treatment with the histone deacetylase inhibitor trichostatin A that apparently activates embryogenesis-related genes such as *LEC1*/*HAP3A* and *ABI3*/*VP1*. Comparison of epigenomic markers in young versus aged cultures could lead to a better understanding of the changes associated with ageing and possibly to the discovery of factors that could be used to modify gene expression and make the cultures productive again (see below *Molecular aspects of somatic and zygotic embryogenesis in pines*).

19.4 Somatic Embryo Development and Maturation

As for other conifers, pine *se* development and maturation were improved on media containing between 40–250 μM of abscisic acid (ABA) depending on the species (Table 19.2). Another critical factor was reduced water availability, which could be imposed by a high gellan gum concentration in the maturation medium to promote

Table 19.2 Somatic embryo yield obtained in *Pinus* spp. using different formulations of maturation medium

Pinus species	Tested lines		ABA (µM)	Sugar (M)/PEG (%)[b]	Gellan gum (g)	*se* yield g^{-1} FW Max.	Reference
	Nb	Maturing[a]					
densiflora	15	11	250	S (0.2)	12	798	Kim and Moon (2014)
halepensis	13	13	75	M (0.16)	9	10–270	Montalbán et al. (2013)
luchuensis	1	1	100	M (0.08)/15	6	282	Hosoi and Maruyama (2012)
nigra	6	5	95	M (0.16)	10	235	Salaj et al. (2014)
oocarpa	2	2	40	M (0.16)/12	6	21	Lara-Chavez et al. (2011)
pinaster	26	15	80	S (0.2)	10	0–274	Humánez et al. (2012)
	39	32	80	S (0.2)	9	0–192	Trontin et al. (2011)
	346	323	80	S (0.2)	9	0–652	Trontin et al. (2016a)
pinea	7	4	121	S (0.17)	10	n/a (low)	Carneros et al. (2009)
radiata	24	24	60	S (0.16)	9	10–1550	Montalbán et al. (2010)
	4	3	212	S (0.16)	3	2–42	Find et al. (2014)
sylvestris	22	20	80	M (0.18)	9	127	Aronen et al. (2009)
	81	57	80	S (0.2)	10	977	Latutrie and Aronen (2013)
strobus x *wallichiana*, F2	261	138	80	M (0.18)	10	350	Daoust et al. (2009)
taeda	5	5	20	M (0.06)/13	2.5	150[c]	Pullman and Johnson (2009)

ABA abscisic acid; *FW* fresh weight; *n/a* not available; *PEG* polyethylene glycol; *se* somatic embryo

[a]Giving rise to cotyledonary *se*

[b]*S* sucrose; *M* maltose

[c]Yield expressed in cotyledonary *se* per ml (proliferation in liquid suspension cultures)

cotyledonary *se* development. This was first demonstrated for *P. strobus* by Klimaszewska and Smith (1997) and then successfully applied to a large number of *Pinus* species (Table 19.2).

19.4.1 Maturation: Improved Yield on mLV but Still Low Genotype Capture

In maritime pine, the best maturation conditions involve EM culture for 12–14 weeks on mLV medium supplemented with 0.2 M sucrose, 80 µM ABA and 9–10 g L^{-1} gellan gum (Lelu-Walter et al. 2006; Humánez et al. 2012; Morel et al. 2014a, b). The switch from EM proliferation to *se* development and maturation has recently been investigated by both transcriptome and proteome profiling (Morel et al. 2014a; Plomion et al. 2016; also, see below *Molecular aspects of somatic and zygotic embryogenesis in pines*).

For initiation and multiplication of embryogenic lines, mLV was confirmed to be a superior formulation compared with mDCR, with a mean maturation yield of ca. 50 cotyledonary *se* g^{-1} FW in a multiyear experiment conducted with 346 embryogenic lines (Trontin et al. 2016a). A majority of the lines (93 %) demonstrated some *se* regeneration capacity (Table 19.2), and a significant subset (61 %) of lines produced mean cotyledonary *se* yield of at least 10 *se* g^{-1} FW. A maximum yield of up to 652 cotyledonary *se* g^{-1} was obtained with some individual lines. However, based on micromorphological observations, only a tiny fraction of early *se* developed to the cotyledonary stage. It has been estimated, on both mDCR (Breton et al. 2006) and mLV (Trontin et al. unpublished results), that only ca. 10 % of early *se* differentiates into cotyledonary *se*. The development of the majority of early *se* is apparently arrested, asynchronous and/or abnormal.

The majority of the embryogenic lines cryopreserved at FCBA from 2000 to 2005 were initiated and propagated on mDCR medium formulations and revealed only poor maturation capacity (mean of ca. 3 cotyledonary *se* g^{-1} FW; Trontin et al. 2016a). One critical question was thus to investigate the maturation capacity of cryopreserved mDCR lines on the more favourable mLV maturation medium. Maturation data were compiled over 15 years (2000–2015) for a model line (PN519; Breton et al. 2006) initiated on mDCR in July 1999 and involved in various maturation experiments with mDCR (2000–2005) and mLV medium (since 2005). The maturation capacity of PN519 drastically and consistently increased on mLV (Fig. 19.6). Mean maturation capacity of PN519 was 12.7 *se* g^{-1} FW on mDCR and 94.0 *se* g^{-1} FW on mLV. Line age since its initiation affected maturation yield on both mDCR and mLV, as previously discussed. In conclusion, PN519 maturation capacity was increased ca. sevenfold on mLV. Interestingly, these data indicate that the maturation capacity of an embryogenic line initiated in suboptimal conditions (mDCR) is not corrupted but may be only revealed on the optimal maturation medium. It seems that the maturation capacity of an initiated line is far from being determined only by the genotype, but also by the culture conditions or other unidentified environmental factors.

Because of the progressive decrease in *se* maturation capacity concomitant with line ageing, genotype capture at the maturation step is particularly difficult to estimate in pines. In maritime pine, maturation yield has been shown to be non-optimal during a period of up to 12 weeks following line thawing from the cryopreserved stock (Breton et al. 2006). Therefore, genotype capture has been

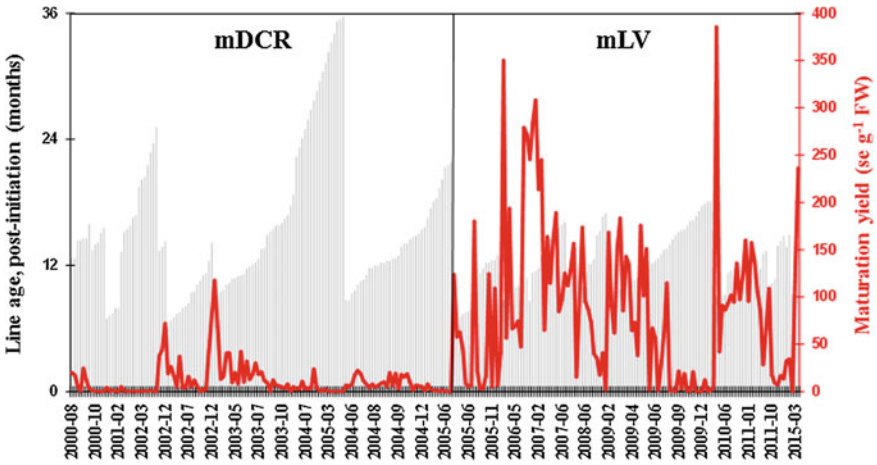

Fig. 19.6 Maturation capacity of a maritime pine embryogenic line (PN519) tested at the FCBA over 15 years (2000–2015) on mDCR or mLV medium (0.2 M sucrose, 80 µM ABA, 9 g L^{-1} gellan gum). The original PN519 line was initiated on July 29, 1999, proliferated, and initially cryopreserved on 8 December 1999 (mDCR media). The line was later re-cryopreserved several times to maintain the cryopreserved stock. Maturation yield (cotyledonary *se* g^{-1} fresh weight/FW) is shown for the experiment involving the original non-cryopreserved line (2000) and then 19 sublines thawed from the cryopreserved stock (2001–2015). The original line and thawed sublines were proliferated and matured on either mDCR (2000–2005) or mLV (2005–2015). mLV medium started being routinely used in 2005 following the results of Park et al. (2006). *Solid bars* (in *grey*) show the chronological line age since initiation (in months) at the time of each maturation experiment (N = 250 data), i.e. the cumulative proliferation time from (i) initiation to cryopreservation (4.2 months) and (ii) line regrowth from the cryopreserved stock to the maturation experiment. *mDCR* modified DCR medium from Gupta and Durzan (1985); *mLV* modified LV medium from Litvay et al. (1985)

redefined as the number of embryogenic lines producing at least 50 cotyledonary *se* g^{-1} FW on mLV medium (Trontin et al. 2016a) after ca. 4 months of proliferation (18 weeks). According to this definition, genotype capture at the maturation step (39 lines tested) was estimated to be 43.6 % (Trontin et al. 2011). Considering only lines yielding at least 100 cotyledonary *se* g^{-1} FW, genotype capture is reduced to 23.1 %. In a considerably larger sample of embryogenic lines matured several times after 2–6 months proliferation on mLV at FCBA (346 lines; Table 19.2), genotype capture was only 28 % (>50 *se* g^{-1} FW) or 16 % (>100 *se* g^{-1} FW).

19.4.2 Conversion of Cotyledonary Somatic Embryo to High-Quality Somatic Seedlings

In maritime pine, cotyledonary *se* are arbitrary harvested after a 12 week culture on a maturation medium (Lelu et al. 1999; Ramarosandratana et al. 2001; Breton et al.

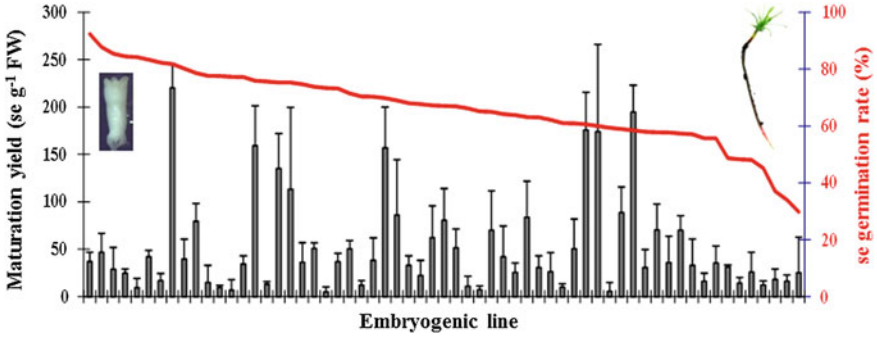

Fig. 19.7 Ranked germination rates (%) of cotyledonary somatic embryos (*se*) and maturation capacity of the corresponding embryogenic lines in maritime pine. *Solid bars*: maturation capacity (*left axis*); red line: germination rate (*right axis*). Data are presented for 61 embryogenic lines (from 23 seed families) initiated at the FCBA and proliferated for 7–12 weeks post-thawing from the cryopreserved stock. EM proliferation and maturation as well as cotyledonary *se* germination were performed on mLV (modified LV medium from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. *FW*: fresh weight. *Bars* represent 95 % confidence limits for the maturation yield

2006; Klimaszewska et al. 2009; Humánez et al. 2012). Biological parameters (dry weight, water content) and biochemical analyses (total protein and carbohydrate contents) showed that cotyledonary *se* harvested after 10 or 14 weeks did not differ markedly and thus confirmed that harvesting cotyledonary *se* after 12 weeks is appropriate (Morel et al. 2014b). Cotyledonary *se* germinated at a high frequency (>70 %, reviewed in Trontin et al. 2016a), although differences were observed among lines. In a sample of 61 productive lines initiated from 23 seed families, mean germination rate of cotyledonary *se* on mLV medium was 66.5 %, with a 30–92 % variation range (Fig. 19.7). Interestingly, germination rate was not correlated with maturation ability (Fig. 19.7) suggesting that *se* development (as judged by cotyledonary *se* yield) and *se* quality (as judged by the germination rate) are not necessarily linked.

Cotyledonary *se* capacity to germinate into plantlets that can be acclimatized in the greenhouse and readily converted into growing somatic seedlings (*ss*) in the nursery was studied at FCBA with six embryogenic lines (Trontin et al. 2016a). The frequency of cotyledonary *se* conversion to *ss* was significantly improved using mDCR germination medium (48.6 %) as compared with mLV (only 34.5 %). The negative effect of mLV on *se* germination rate and conversion to plants could be already determined during the maturation phase (Trontin et al. 2016a), suggesting that the quality of harvested *se* is suboptimal.

Zygotic embryos and/or seedlings were introduced as the reference standard for estimating initial growth and vigour of cotyledonary *se* harvested after 12 weeks of maturation on the best maturation conditions for maritime pine (mLV, 0.2 M sucrose, 80 µM ABA and 9 g L^{-1} gellan gum). As a very important limitation to SE implementation in breeding programmes, *se* growth post-acclimatization is far

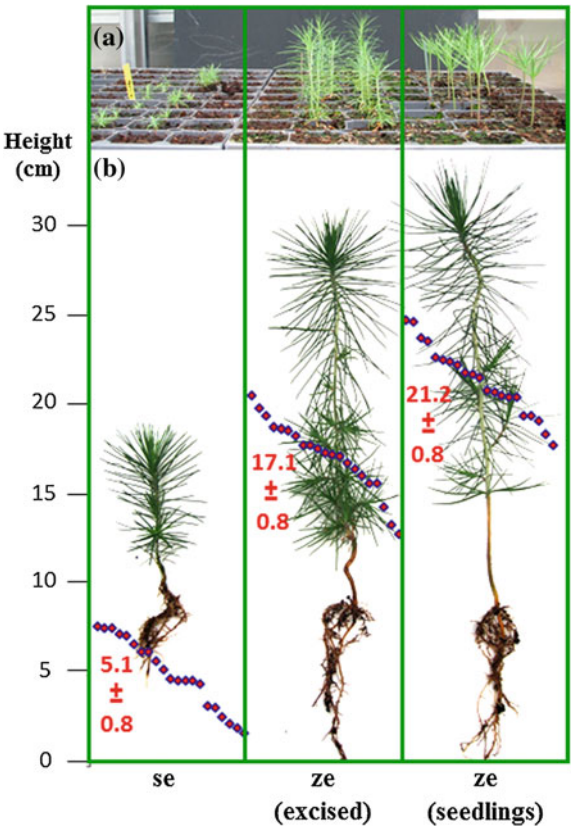

Fig. 19.8 Growth behaviour (shoot height) of somatic seedlings from a maritime pine embryogenic line (NL04045) and excised *ze* from mature seeds or seedlings from the corresponding NL full-sib family after 6 (A) or 22 weeks (B) of development (*se*, excised *ze*), or post-germination (seedlings). Cotyledonary *se* and excised *ze* were germinated in vitro in the same conditions for 10 weeks before acclimatization (mLV medium). Corresponding seedlings were sown at the time of *se*/excised *ze* acclimatization after stratification for 5 weeks at 4 °C. Mean heights ±95 % confidence limits (in *red*) and individual height (*red dots*, N = 21 plants) are both presented in panel B. Observed differences between means are significant (t-tests, $\alpha = 0.05$)

from matching that of seedlings and is significantly lower than dissected *ze* germinated under the same in vitro conditions as cotyledonary *se*. As an example, Fig. 19.8 shows the shoot height of *ss* from one embryogenic line after 22 months of growth in the greenhouse and the corresponding height of dissected *ze* from mature seeds and seedlings from the same family. Both mean height and individual height of *ss* are below that of control family. The striking difference observed with dissected *ze* shows that the intrinsic quality of cotyledonary *se* is not appropriate to obtain high initial vigour that is similar to that of seedlings. Several field trials established with *ss* and control seedlings confirmed the initial low growth rate of somatic clones in maritime pine (Trontin et al. 2016a; also, see below *Field performance of the se-derived pines*). These results have huge practical implications for refining the maturation conditions in maritime pine towards the production of high-quality somatic seedlings.

For the production of *ss* of eastern white pine hybrids the most critical factor was to synchronize the acclimatization in a greenhouse with the beginning of the natural vegetative season and the application of suitable fertilization regime (Fig. 19.9, Klimaszewska et al. unpublished).

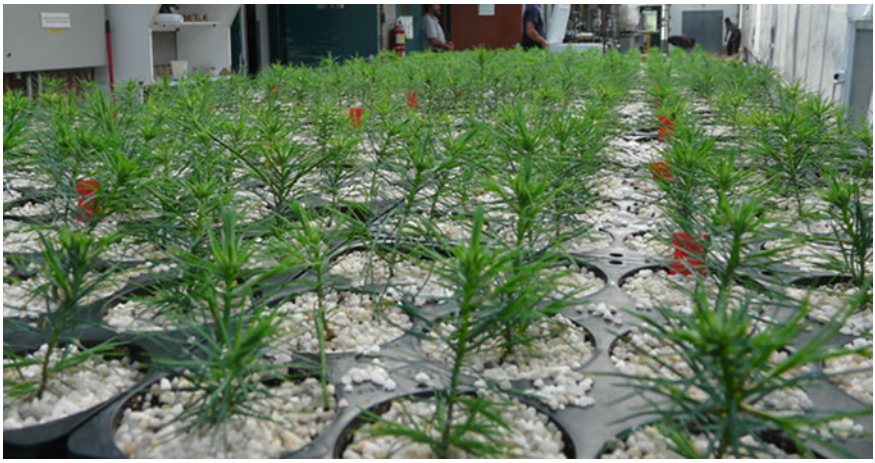

Fig. 19.9 Somatic seedlings of *Pinus strobus* x *P. wallichiana* F2 hybrids produced for the selection of white pine blister rust tolerance at the Laurentian Forestry Centre (CFS-NRCan) Quebec, Canada

19.5 Molecular Aspects of Somatic and Zygotic Embryogenesis in Pines

Although impressive advances have been made in the establishment of SE in *Pinus* spp. (see above *Induction of somatic embryogenesis in Pinus* spp.), further progress is expected concomitant with a better understanding of the molecular mechanisms of embryo development and pattern. This fundamental knowledge is important to achieve and/or further optimize all stages of SE, from induction to *se* maturation and *ss* regeneration (Miguel et al. 2016). With these goals in mind, several approaches and methodologies targeting different layers of molecular information have been applied in conifers (Trontin et al. 2016b). Genes, transcripts, proteins, metabolites and epigenetic mechanisms have been analyzed. Comparative analyses between SE and zygotic embryogenesis (ZE), regarded as the model for SE, have highlighted important processes during embryo development. Moreover, molecular profiling studies conducted either with *se* or *ze* (or comparative studies of both) have generated basic knowledge on embryo developmental pathways that could be used to optimize SE. Transfer of knowledge from more efficient conifer SE systems, e.g. *Picea abies*, has also contributed to the recent progress (von Arnold et al. 2016).

However, these efforts have been hindered by the intrinsic characteristics of these long-lived species, which include huge genome sizes and, consequently, the lack (until recently) of large and comprehensive molecular resources compared with model angiosperm species. Specifically, since reverse genetics approaches are mostly based on SE for producing mutant lines and plants, their success largely depends on the availability of an effective SE system for the species under study.

Despite these obstacles, progress has been reported from targeted and genomewide studies (transcriptomics, proteomics and metabolomics) and much more is expected in the coming years, as the genome sequences of several pine species (e.g. *P. taeda*, *P. radiata* and *P. pinaster*) are being decoded and released (Plomion et al. 2016).

19.5.1 Molecular Markers to Evaluate Genetic Stability

The first molecular studies of SE in conifers, including pines, were performed with the aim of evaluating genetic stability of the cultures (Fourré 2000). The occurrence of genetic alterations, which may include changes in chromosome number and/or structure and in DNA sequence, is one of the most often suggested causes for loss of *se* maturation capacity in aged conifer EM and for deviation from normal embryo development. Such alterations may affect the function or regulation of genes involved in embryogenesis, potentially leading to variable success at different SE stages or to abnormalities in the *se* phenotypes, and have been suggested to be an adaptation response to the stress imposed either by in vitro culture or by environmental conditions in general, thus reflecting plant developmental plasticity. Therefore, investigation of genetic instability and its putative association with embryo or EM developmental characteristics has been performed in several *Pinus* species using DNA markers, karyological and flow cytometric analyses (Burg et al. 2007; Marum et al. 2009a, b; Miguel et al. 2016).

Due to their ubiquity in genomes and high mutability, simple sequence repeats (SSRs, or microsatellites) are considered sensitive markers for monitoring putative mutation events in cultured cells. The analysis of four nuclear microsatellite loci during the establishment, proliferation and maturation of embryogenic cultures of *P. sylvestris* made it possible to conclude that genetic instability within family correlated positively with embryogenecity (the ability to establish embryogenic cell lines), but negatively with the frequency of cotyledonary embryo formation (Burg et al. 2007). Although the variability was higher during SE in four out of ten families, *ze* also showed some variation, suggesting that instability in the analyzed SSRs might reflect the plasticity of the family during adaptation to environmental conditions. As pointed out by the authors, it would be important to further investigate whether instability reflects alteration in functional genes that are somehow involved in embryogenecity or in the embryo developmental process.

Putative correlations between genetic instability and abnormal embryos or *ss* morphology were also investigated in *P. pinaster* (Marum et al. 2009a, b). While no major changes were detected in ploidy level as analyzed by flow cytometry, even in *se* with abnormal phenotype (Marum et al. 2009a), variation in SSR loci was found in both proliferating EM lines and *ss*. However, genetic instability in the analyzed loci could not be correlated with abnormal *ss* phenotype (Marum et al. 2009b). Due to the lack of clear conclusions from these and other studies in conifers (reviewed in Miguel et al. 2016), the effectiveness of such approaches to monitor SE is still a matter of debate.

19.5.2 SE Induction and EM Multiplication

The molecular studies focusing on SE induction and EM propagation that have been conducted in pines were based on either targeted or genomewide expression analyses and were mostly aimed at the identification of markers with embryogenic potential (Table 19.3). *LEC*, *SERK* and *ABI3/VP1* well-known genes putatively coding for transcription factors such as the *WUSCHEL* (*WUS*)-related homeobox (*WOX*) family and associated with plant embryogenesis, particularly with SE induction (Elhiti et al. 2013; Mahdavi-Darvari et al. 2015), have been investigated. Transcript expression profiling in *P. radiata* shoot-derived calli and ze-derived EMs showed correlation of *WOX2* and *ABI3* expression with embryogenic potential whereas *LEC1* was expressed in both EM and non-embryogenic calli (Garcia-Mendiguren et al. 2015). This is consistent with previous studies conducted in other conifer species (Palovaara and Hakman 2008; Klimaszewska et al. 2011), including *P. contorta* where *PcWOX2* was expressed in EM from immature ze and EM-like tissues derived from mature trees but not in non-embryogenic calli derived from a seedling needle (Park et al. 2010). Also, *ABI3/VP1* appeared to be expressed at low levels during EM proliferation in several conifer species including *P. sylvestris* (Uddenberg et al. 2011). On the other hand, a *LEC1*-like transcript (*PcHAP3A*) in *P. contorta* appeared to be mainly associated with non-embryogenic calli, but it was also expressed at a high rate in EM (Park et al. 2010), while in *P. sylvestris*, *LEC1/PsHAP3* was highly expressed during early SE or ZE (Uddenberg et al. 2011). Expression of a *SERK1* gene was similarly detected in proliferating EMs from *P. massoniana* and also at later embryo developmental stages (Yan et al. 2010). Given their different expression patterns in different conifer species as reported in functional studies (Klimaszewska et al. 2010, 2011; reviewed in Trontin et al. 2016b; Miguel et al. 2016), the role of these genes in embryogenesis remains unclear. Other targeted gene and transcriptomic studies in *P. radiata* have also highlighted some additional genes that, according to their differential expression in embryogenic versus non-embryogenic tissues or during SE, might play important roles in specific stages of the process (Bishop-Hurley et al. 2003; Aquea and Arce-Johnson 2008; Aquea et al. 2008) such as a group of five gene families (Table 19.3) that includes four putative extracellular proteins (Bishop-Hurley et al. 2003).

19.5.3 Somatic Embryo Development and Maturation

The switch from proliferation of EM to embryo maturation represents one of the most challenging and critical steps of SE in pines, being dependent on the genotype, culture conditions and the age of the cultures. It has become evident that molecular studies can prove valuable in understanding the effect of these factors and optimizing SE accordingly.

Table 19.3 Recent studies focusing on the molecular aspects of somatic and zygotic embryo development in *Pinus* spp.

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
P. contorta	EM, EM-like, NET	qRT-PCR	*PcWOX2*, *LEC1* (*PcHAP3a)*	*WOX2* specifically expressed in EM and EM-like obtained from primordial shoot explants of mature tree	Park et al. (2010)
P. massoniana	Early-late *ze*	2-DE DIGE, MS/MS	Proteome	Proteins associated with *ze* stages up to the coty. stage	Zhen et al. (2012)
	Early-late *se*	qRT-PCR	*PmSERK1*	*PmSERK1* is expressed in proliferating EMs and coty. *se*	Yan et al. (2010)
P. nigra	EM	HPCE HPLC	Genomic DNA methylation Free polyamines level	Low % 5-mdC and low free polyamines associated with high embryogenic capacity of EMs	Noceda et al. (2009)
P. oocarpa	Early-late *se*	qRT-PCR	*Legumin-like*, *vicilin-like*, *LEA*, *HD-Zip I*, *26S proteasome subunit S2* (*RPN1*), *clavata-like*.	*P. oocarpa* versus *P. taeda se*: variation of *legumin*-like, *vicilin*-like, *LEA and HD-Zip I* expression occurred at late coty. stages; d*issimilar RPN1* and *Clavata-like* expression; lower *se* quality in *P. oocarpa*	Lara-Chavez et al. (2012)
P. pinaster	Early-late *se*, *ze*, seedlings	*In situ* hybridization, northern blot	*Glutamine synthase (GS1a*, *GS1b)*, *Arginase*	*GS1b* associated with vascular pattern formation *Arginase* expressed in coty. *se* but not in *ze*	Pérez Rodríguez et al. (2006)
	Early-late *ze*, seedlings	qRT-PCR, *in situ* RT-PCR	Rab-related small GTP-binding protein (*PpRab1*)	*PpRab1* upregulated at the early *ze* stages	Gonçalves et al. (2007)
	Young versus aged EMs	HPCE HPLC MSAP	Genomic DNA methylation Hormones/polyamines level Methylation pattern	5-mdC level not associated with EM ageing Inconsistent hormones/polyamines levels EM ageing associated with net (de) methylation	Klimaszewska et al. (2009)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Mature *se*, *ze*	HPCE	Genomic DNA methylation	Lower 5-mdC level in abnormal *se*	Marum (2009)
	Early-late *ze*	qRT-PCR	Alpha-Amylase Inhibitors Lipid Transfer Seed Storage protein (*PpAAI-LTSS1*)	*PpAAI-LTSS1* expressed in pre- and early coty. *ze*	Simões et al. (2011)
	Early-late *se*, seedlings	qRT-PCR, genetic transformation	*Clavata1-like*	*Clavata1-like* may play a role in caulogenesis Promoter drives expression from early to late *se* development	Alvarez et al. (2013)
	Early-late *ze*	cDNA microarray	25848 cDNA clones	Most changes at transitions from early to pre-coty. and from coty. to mature *ze*; epigenetic (transposable elements, histone modification, small/microRNA) and auxin-mediated regulation (transcription factors) of *ze* development.	de Vega-Bartol et al. (2013)
	Early *se*	RNA-seq (Illumina) 2-DE, MS/MS	Transcriptome Proteome	Unfavourable maturation conditions (4 g L^{-1} gellan gum) enhance glycolytic pathways, resulting in cell proliferation Favourable conditions (9 g L^{-1}) activate protective pathways and ABA-mediated responses, promoting *se* development	Morel et al. (2014a)
	Late *se*, *ze*	HPLC, Bradford test 1/2-DE, MS/MS	Carbohydrates/proteins level Proteome	Coty. *se* matured for 10-14 weeks are similar Coty. *se* are similar to fresh, undesiccated coty. *ze* 23 protein markers of fresh coty. *se*/*ze* stage	Morel et al. (2014b)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
P. pinea	Seedlings	qRT-PCR	*Clavata1-like*	*Clavata1-like* may play a role in caulogenesis	Alvarez et al. (2013)
P. radiata	Early-late *se*, NET	cDNA library, RT-PCR, northern blot	28 embryogenesis-related cDNA clones	*Germin*, *α-expansins*, *21 kDa precursor*, *cytochrome P450* and an unknown gene (*PRE87*) expressed from early to late *se* development.	Bishop-Hurley et al. (2003)
	Early *se*, NET (seedlings)	cDNA-AFLP, qRT-PCR	Otubain-like cystein protease (*PrOTUBAIN*)	*PrOTUBAIN* highly and preferentially expressed in embryogenic tissue	Aquea et al. (2008)
	Early *se*, NET (needles)	cDNA-AFLP, RT-PCR	4000 transcript-derived fragments (TDFs)	50 TDFs upregulated (cellular metabolism, stress response) and 32 TDFs downregulated (proteolysis, cell wall modification, signalling pathways) in early *se*	Aquea and Arce-Johnson (2008)
	Early-late *se*	qRT-PCR	GRAS family (*PrSHR*, *PrSCL1* and 13 other GRAS genes)	GRAS gene expression is high in coty. *se* Increased expression of *PrSHR*, *PrSCL1* and five other GRAS genes at the beginning of *se* differentiation	Hernández et al. (2011)
	EM, NEC	qRT-PCR	*LEC1*, *WOX2*, *ABI3*, *Histone 4*, *PCNA*, *SKN1-4*, *WOX4*	Putative embryogenesis-related *LEC1* is expressed in NEC from axillary primordial shoot explants of mature tree *WOX2* and *ABI3* are associated with embryogenecity	Garcia-Mendiguren et al. (2015)
P. strobus	Late *se*, *ze*, seedlings	1-DE, MS/MS	Storage proteins	Most abundant proteins are 11S-globulin and 7S vicilin-like Less storage proteins accumulated in *se* versus *ze* (11S-globulin)	Klimaszewska et al. (2004)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
sylvestris	Early-late *se*, *ze*, seedlings	*In situ* hybridization, northern blot	*GS1a*, b (*glutamine synthase*), *rbcs (rubisco small subunit), psbO (33 kDa protein of PSII)*	*GS1b* associated with vascular pattern formation *GS1a, rbcs* and *psbO* expressed in coty. *se* but not *ze*	Pérez Rodríguez et al. (2006)
	Early-late *ze*	qRT-PCR, *in situ* hybridization, HPLC	Polyamines levels, *Arginine decarboxylase* (*ADC*)	Polyamines content increases in early *ze* and decreases in late *ze* (except free putrescine fraction); *ADC* expression increases from early to late *ze*	Vuosku et al. (2006)
	Early-late *se*, NEC	RT-PCR, gas chromatography	*1-aminocyclopropane-1-carboxylate synthase* (*PsACS1*, *PsACS2*),	*PsACS2* expressed only at the maturation step and correlated with both ethylene production and higher *se* maturation yield	Lu et al. (2011)
	Early-late *se*, *ze*	qRT-PCR	*LEC1/PsHAP3A/PsHAP3B*, *ABI3/PsVP1*	*PsHAP3A* expression is high in EMs and early *ze* *PsVP1* expression increases with *se*/*ze* development	Uddenberg et al. (2011)
	Early-late *ze*, FG	qRT-PCR, *in situ* hybridization	*Autophagy related ATG5*, *retinoblastoma related* (*RBR*), *catalase* (*CAT*),	*ATG5*, *CAT* and *RBR* are involved in embryo development and cell death processes	Vuosku et al. (2015)
taeda	Early-late *se*, *ze*	cDNA array, differential display	326 cDNA clones	Evidence for difference in gene expression between early and late *se* and between *se* and *ze*	Cairney et al. (2000)
	Early-late *se*, *ze*, seedlings	Northern blot, RT-PCR	*Aquaglyceroporin* (*PtNIP1;1*)	*PtNIP1;1* preferentially expressed in suspensor tissue	Ciavatta et al. (2001, 2002)
	Early-late *se*, *ze*	cDNA array	326 cDNA clones	Gene expression in coty. *se* is most similar to early coty. *ze*	Pullman et al. (2003)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early-late *se*, *ze*	Biochemistry, 1-DE	Triacylglycerol and storage protein contents	Triacylglycerol increases during se maturation but is lower than in *ze*; higher storage (soluble) proteins content in *se*	Brownfield et al. (2007)
	Late *se*, *ze*	cDNA array	326 cDNA clones	Increased ABA during maturation makes the *se* mRNA profile more like *ze* for *starch synthase*, *small HSP*, *HSP70*, *LEA* (x2), *XETG-like*, *40S ribosomal protein*, *cyclic phosphodiesterase* and four unknown genes	Vales et al. (2007)
	Early *se*, early-late *ze*, FG, seedlings	Northern blot, qRT-PCR	AGO genes (miRNA metabolism, *PtAGO1,9L*), five micro RNAs and putative target: *miR159/MYB33*, *miR166/class III HD-ZIP*, *miR167/ARF8*, *miR171/scarecrow* and *miR172/apetala 2*	*miR166* detected in early *se* but not *miR167* Stage-specific modulation of five miRNA in FG and *ze* *PtAGO1L* and *PtAGO9L* are stage-specific and inversely regulated in *ze* and FG The peak levels of *miR166* in FG and *ptAGO9L* in coty. *ze* occurred at a critical transition point where *se* maturation often stops	Oh et al. (2008)
	Early-late *se*, *ze*	Semi quantitative RT-PCR	*Serine palmitoyltransferase* (*PtSPT1, PtSPT2*), *ceramide kinase* (*PtCERKL*, *PtCERKS*)	*PtSPT1* expression is lower in *se* than in *ze*, and either constant (*se*) or increasing (*ze*) from early to late embryogenesis Only *PtCERKL* expressed in *se* and in a gradually increasing pattern, whereas expression is constant from early to late *ze*	Zhu (2008)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early *se*	GC/MS	Metabolome	The regenerative capacity of an embryogenic line can be accurately predicted using a subset of 47 metabolites related to nutrient uptake/allocation at the transition to maturation and response to stress during proliferation Primary metabolites with the strongest influence in the model are sucrose, glutamine, fructose-6-phosphate, dehydroascorbate, malic acid, an unidentified purine, threonine, asparagine, glycine, serine and proline	Robinson et al. (2009)
	Mid-late *ze*	qRT-PCR	ABA-responsive genes (*ABI3,4,5*) and six genes involved in root development: *woodenleg* (*PtWOL*), *short root (PtSHR)*, *hobbit (PtHBT)*, *bodenlos (PtBDL)*, *scarecrow(PtSCR)* and *monopteros* (*PtMP*)	*ABI3,4,5* exhibited a three-phase pattern of expression in coty. *ze* that may relate to an oscillating pattern of sugar accumulation *PtSCR* and *PtSHR* are overexpressed at the time of cell differentiation around RAM in coty. *ze* *PtBDL*, *PtMP*, *PtHBT* and *PtWOL* specifically expressed at the time of establishment of germination competence	Jones (2011)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early-late *se*, *ze*	qRT-PCR	*Legumin-like*, *vicilin-like*, *LEA*, *HD-Zip I*, *26S proteasome subunit S2* (*RPN1*) and *clavata-like*	Gene expression similarities between *se* and *ze* Higher gene expression in *se* (except *LEA*) may be attributed to continual exposure to ABA *P. taeda se* are of higher quality than those of *P. oocarpa*	Lara-Chavez et al. (2012)

[a]*EM* embryonal mass; *FG* female gametophyte; *NET* non-embryogenic callus (NEC) or vegetative tissue; *se* somatic embryo; *ze* zygotic embryo
[b]*AFLP* amplified fragment length polymorphism; *cDNA* complementary DNA; *1/2-DE* one/two-dimensional gel electrophoresis; *DIGE* difference gel electrophoresis; *GC/MS* gas chromatography coupled with mass spectrometry; *HPCE* high-performance capillary electrophoresis; *HPLC* high-performance liquid chromatography; *MSAP* methylation-sensitive amplified polymorphism; *MS/MS* tandem mass spectrometry; *PSII* photosystem II; *qRT-PCR* reverse transcription quantitative polymerase chain reaction; *RNA-seq* RNA sequencing
[c]*Coty.* cotyledonary; *5-mdC* 5-methyldeoxycytidine

Recent studies in pines and other conifers using different molecular approaches, predominantly at the transcript level, pointed to major pathways that are essential to normal *se*/*ze* development (Trontin et al. 2016b). These include developmentally regulated programmed cell death (PCD), megagametophyte function and/or signalling, cell wall modifications, auxin response machinery and other important regulators of embryo patterns including epigenetic regulation, ABA-mediated developmental switch, changes in carbohydrates, proteins and energy metabolism, stress-related responses and the maintenance of redox homeostasis (Table 19.3).

A striking example of the importance of molecular approaches for understanding the effect of culture conditions on SE is the analysis of water availability during embryo maturation. Embryo maturation in pines is drastically affected by the gel strength of the culture medium, which determines water availability. In *P. strobus*, *P. sylvestris* and *P. pinaster*, reduced water availability (9–10 g L^{-1} gellan gum) results in the formation of cotyledonary *se* within 12 weeks, which are able to germinate (Klimaszewska and Smith 1997; Klimaszewska et al. 2000; Ramarosandratana et al. 2001; Lelu-Walter et al. 2006, 2008; Aronen et al. 2009), while increased water availability (4 g L^{-1} gellan gum) leads to the excessive proliferation of EM, thereby interfering with embryo maturation (Morel et al. 2014a). In *P. pinaster*, significant differences between both conditions were detected at the molecular level after only 1 week of culture on the maturation medium (Morel et al. 2014a). Under increased water availability, the hexose/sucrose ratio was high in the proliferating EM, suggesting stimulation of the glycolytic pathway, corroborated by both transcriptomic and proteomic analyses. Also, proteins involved in cell division and DNA replication, such as DNA replication licensing factor MCM3 homolog 3 and cell division protein FtsQ, were overexpressed, in accordance with the transcriptomic studies and fresh weight increases. One of the most significantly overexpressed proteins was related to a 26S proteasome regulatory subunit involved in the selective breakdown of proteins and could be an important player in the regulation of cell senescence, in agreement with the necrotic cells observed as early as 2–3 weeks after the beginning of maturation. On the other hand, under reduced water availability, gene expression was apparently reoriented through genomewide mechanisms such as chromatin modification (high expression of various ubiquitin protein ligase genes involved in the activation of the small ubiquitin-related modifier) and de novo protein biosynthesis from controlled proteolysis (expression of proteasome subunit beta 1 proteins, ubiquitin/26S proteasome pathway). Concomitantly, a major increase in endogenous ABA occurred after 4 weeks of maturation and is thought to be an ontogenetic signal for ABA-mediated molecular and physiological responses promoting embryo development. After only 1 week of maturation, an upregulation of transcripts related to ABA-mediated molecular response (high expression of protein phosphatase 2C and serine/threonine kinase genes; see also Bishop-Hurley et al. (2003) for *P. radiata*) occurred, as well as an activation of protective pathways such as synthesis of secondary metabolites (flavanone 3-hydroxylase, flavonol metabolism; Morel et al. 2014a) or other defence genes (see Aquea and Arce-Johnson (2008) for *P. radiata*).

At the proteomic level, many overexpressed proteins showed control of the proteasome-dependent proteolysis pathway of PCD (disulfide isomerase, chitinase), of the regulation of oxidative stress and maintenance of cell redox homeostasis (superoxide dismutase proteins, germin-like proteins and genes; see also de Vega-Bartol et al. (2013) for *P. pinaster*, Bishop-Hurley et al. (2003) and Aquea and Arce-Johnson (2008) for *P. radiata*, and Pullman et al. (2015) for *P. taeda*), of the cell division oriented towards cell remodelling (expansins, tubulin beta-2, GTP-binding nuclear protein Ran-1), and of ABA-mediated molecular responses. Overexpression of various proteins (glucose-1-phosphate adenylyltransferase AGPP, 4-alpha-glucano-transferase DPE2, disproportioning enzyme) also suggested an activation of the biosynthesis of starch granules (Morel et al. 2014a). Starch accumulation has been associated with early embryo development in maritime pine (see Tereso et al. 2007). A chart representing early physiological, cellular and molecular events during SE in maritime pine, in the presence of either low or high gellan gum concentration (Morel et al. 2014a), can be found in Plomion et al. (2016).

A proteomics study of early ZE in *P. massoniana* (Zhen et al. 2012) also revealed that some of these large functional molecular classes were successively overexpressed during seed development, including in the cleavage polyembryony, dominant embryo, columnar embryo and early cotyledonary embryo stages. Proteins involved in carbon and energy metabolism were overrepresented at the cleavage polyembryony and columnar embryo stages, which is consistent with a higher energy and carbohydrate requirement, and were also possibly involved in starch synthesis. A significant amount of proteins required for embryo morphogenesis seem to be then synthesized and assembled under the control of chaperones and heat shock proteins. This intense activity during embryo morphogenesis is accompanied by an essential regulation of the redox cell system (Zhen et al. 2012).

A well-known limitation of *Pinus* SE is the variation in the capacity of EM to produce cotyledonary *se*, i.e. embryo maturation capacity as a function of the genotype or other factors such as ageing. Ageing of EM during the subculture process has been pointed out as one of the main factors leading to the systematic loss of maturation capacity (Breton et al. 2006; Klimaszewska et al. 2009). In maritime pine, Marum et al. (2009b) detected variations at 3 SSR loci after proliferation for 6, 14 and 22 months in two out of 17 embryogenic lines. However, logistic regression did not reveal any significant effect of the number of subcultures on mutation rate. The apparent lack of association between accumulation of genetic mutations and maturation ability is consistent with the results obtained at FCBA for this species. Strikingly, none of the ca. 2,000 embryogenic lines (genotypes) tested in the past 15 years were stable over time during proliferation (i.e. the loss of maturation ability is general) in contrast to what is observed in some other conifers (e.g. *Picea*, *Larix*). Epigenetic mechanisms are probably involved since it is rather unlikely that mutations should systematically affect the same genes involved in maintenance of *se* regeneration capacity in different genotypes. Moreover, in maritime pine, it was demonstrated that spatial separation (in different Petri dishes) of EM sublines (from the same genotype) with different maturation capacity had erratic consequences during subsequent subcultures on the maturation capacity

which changed from low to high yield or vice versa (Breton et al. 2006). Random, reversible changes in *se* maturation yield are strongly indicative of environmentally induced modifications of gene expression. Epigenetic variations can be induced by culture conditions through various mechanisms involving DNA methylation, histone modifications and chromatin remodelling as well as expression of small RNAs (Miguel and Marum 2011). Such processes are interconnected and are the part of the epigenetic complex of regulation of gene expression, in particularly that of embryogenesis-related genes involved in maintenance of the embryogenic state and regeneration capacity (reviewed in Trontin et al. 2016b; Miguel et al. 2016).

In an attempt to identify the underlying causes of this phenomenon, several studies have been conducted based on biochemical analyses and DNA methylation (discussed in the section below). The levels of polyamines or hormones were analyzed in lines with varying capacity for embryo maturation in *P. pinaster* (Klimaszewska et al. 2009) and *P. nigra* (Noceda et al. 2009). While inconsistent polyamine and hormone profiles were displayed by EM cultures of different genotypes with similar maturation capacity in *P. pinaster*, an inverse relationship between total contents of free polyamines and maturation capacity was reported in *P. nigra*. Polyamine and hormone profiles were also compared between young (productive) and aged EM (non-productive) of the same genotype. No difference could be detected for one genotype, whereas a second genotype showed a higher level of indole-3 aspartate and lower levels of zeatin riboside, free spermidine and spermine in young EM cultures (Klimaszewska et al. 2009). Interestingly, in *P. taeda*, a subset of 47 metabolites identified by GC/MS-based metabolomics of propagated embryogenic lines correlated with maturation capacity and can be used as a predictive screening tool because the corresponding model is largely independent of the genotype (Robinson et al. 2009).

19.5.4 Epigenetic Analyses Throughout se Development

Epigenetic mechanisms have emerged as being critical in the control of both SE and ZE in different plant species (Nodine and Bartel 2010; Miguel and Marum 2011; De-la-Peña et al. 2015; Trontin et al. 2016b) by ultimately determining gene expression patterns through modulation of access to DNA and definition of distinct chromatin states. Chromatin remodelling may occur during proliferation and early embryo development in pine as observed in *P. sylvestris* (Uddenberg et al. 2011) and *P. pinaster* (de Vega-Bartol et al. 2013; Morel et al. 2014a).

A special attention has been given to DNA methylation analysis, as a number of tools for detecting this type of variation are readily available (Miguel and Marum 2011). Noceda et al. (2009) found that the global methylation levels analyzed in *P. nigra* embryogenic cell lines gradually decreased with the increased maturation capacity of the line, varying from 18 to 30 % 5-methyldeoxycytidine (5-mdC) in the lines showing the highest (80 mature *se* g^{-1} EM FW) and null maturation capacity, respectively. This type of study suggests that demethylating agents used to

manipulate the DNA methylation status might also be used to improve the regeneration capacity of pine embryogenic cultures. However, when testing this hypothesis in *P. pinaster* EM, Klimaszewska et al. (2009) found inconsistent changes in DNA methylation in the cultured EM. Treatment of aged EM with the hypomethylating drug 5-azacytidine led only to a slight increase in maturation capacity. The analysis of both global DNA methylation and methylation patterns as detected by MSAP (methylation-sensitive amplification polymorphism) in EM cultures of different ages (i.e. different regeneration capacity) revealed an association of ageing with net DNA demethylation or methylation at specific target sequences, but not with global DNA methylation levels, thus suggesting discreet instead of large epigenetic changes. In another study, global DNA methylation was reported to vary among genotypes but it could not be related with *se* maturation capacity (Trontin et al. 2016a). Also in *P. pinaster*, Marum (2009) observed that the relative percentages of 5-mdC quantified by HPCE both in mature *se* and *ze* as well as in derived plants were very similar (23–24 % 5-mdC for embryos and 17 % for plants). However, mature *se* with abnormal phenotype presented 3.5 % less 5-mdC when compared with *se* of normal phenotype.

The relevance of several genes involved in the maintenance of chromatin silencing, regulation of histone acetylation or methylation and regulation of DNA methylation has also been highlighted by functional category analysis of differentially expressed genes in *P. pinaster* ZE (de Vega-Bartol et al. 2013). During early embryogenesis, gene silencing mechanisms seem more active, probably to control transposable elements, while from mid to late embryogenesis stages, upregulation of several putative chromatin-remodelling ATPases (CHC1, RAD5 and BSH) was detected. Additionally, differential regulation of several transcripts with homology to known regulators of small RNA biogenesis, processing, and function was identified across all stages of pine embryo development. Genes involved in micro RNA (miRNA) biogenesis and various miRNAs were specifically modulated at different development stages of *ze* and in female gametophyte in *P. taeda* (Oh et al. 2008). Some miRNAs were also regulated during early SE, particularly *miR166* targeting a *class III HD-Zip* transcription factor gene apparently involved in the critical transition point during late ZE where *se* maturation often stops in *P. taeda*.

19.5.5 Comparative Molecular Analyses for Evaluation of Embryo "Quality"

In order to obtain high-quality embryos, leading to vigorous *ss*, it is essential to optimize the maturation conditions, including the duration of the treatment before the beginning of germination. Embryos are usually harvested based on morphological criteria that do not necessarily reflect their maturation status or "quality".

When characterizing the relative expression of six developmentally regulated genes during ZE in *P. taeda* and *se* development/maturation in *P. taeda* and *P. oocarpa*, Lara-Chavez et al. (2012) showed that expression levels usually tended

to be higher in *P. taeda se* compared with similar *ze* stages. Such difference may be attributed to continuous exposure to ABA during SE. Additionally, differences in the expression profile of several genes involved in late maturation (e.g. coding for storage proteins) were consistent with a higher similarity of late cotyledonary *se* to mature *ze*, confirming the empirical choice of that stage for germination (Lara-Chavez et al. 2012). Furthermore, they suggested that the differences in transcript levels observed at late stages of cotyledonary *se* between *P. oocarpa* and *P. taeda* could explain the low germination success and overall lower quality of *P. oocarpa se*.

Expression of genes normally induced in a shoot pole during germination (*arginase*) or in photosynthetic tissue (*glutamine synthase/GS1a*, *rubisco small subunit/rbcs*, *33 kDa protein of photosystem II/psbO*) was detected in cotyledonary *se* but not in *ze* of *P. pinaster* or *P. sylvestris*, suggesting precocious germination and therefore inferior quality of *se* (Pérez Rodríguez et al. 2006). These results suggest that cotyledonary *se* did not undergo the desiccation-induced dormancy that normally occurs in a seed and which separates the embryo maturation stage from germination and post-germination growth (see Pérez Rodríguez et al. 2006 and references therein).

The biochemical composition of *ze* during late maturation has also been investigated in an attempt to define the optimal timing for transferring cotyledonary *se* to a germination medium (Brownfield et al. 2007; Vuosku 2011; Morel et al. 2014b; Pullman and Bucalo 2014). The type and contents of proteins, sugars, ethylene and polyamines were suggested to be critical for *se* post-maturation stages affecting *ss* quality.

During embryogenesis there is a typical trend towards an increase in the levels of spermine and spermidine during early developmental stages and a decrease of these levels during late embryo development. Putrescine remains relatively stable throughout embryo development. Therefore, the spermidine/putrescine ratio has been proposed to follow maturation in *P. sylvestris* (Vuosku et al. 2006).

Considering protein analyses, the same storage proteins or their coding transcripts have been identified in the *se* of *P. strobus* (Klimaszewska et al. 2004), *P. pinaster* (Morel et al. 2014b), *P. taeda* and *P. oocarpa* (Lara-Chavez et al. 2012), and have been shown to accumulate in different conifer species from precotyledonary to (mainly) cotyledonary stages (Lippert et al. 2005; Lara-Chavez et al. 2012). In *P. sylvestris*, it has also been reported that storage proteins were present in equivalent amounts between mature *se* and *ze* (Lelu-Walter et al. 2008).

In *P. pinaster*, no differences were observed in cotyledonary *se* after 10–14 weeks of maturation with respect to contents of total proteins, various mono- or polysaccharide sugars, water and dry matter (Morel et al. 2014b). When 12-week-old *se* were compared with *ze* at different stages of maturity (fresh cotyledonary to desiccated cotyledonary stages) using the same molecular and other biological and physiological parameters, it was clear that they corresponded the most to the fresh, maturing cotyledonary *ze* found in green cones that are present in France in late July/early August. Genomewide proteomic profiling of both *se* and *ze* revealed 94.5 % similarity of detected proteins. At this stage, *ze* just completed

their morphological development (histodifferentiation, embryo patterning). As stated above, this work further confirmed that cotyledonary *se* successfully completed morphogenesis under the current "optimized" maturation conditions, but that they only partially executed the further desiccation process that should result in fully mature *se*. A similar result was reported in *P. taeda* following cDNA macroarray profiling of cotyledonary *se* and *ze* (Pullman et al. 2003). Differences between late *se* and *ze* were also apparent after profiling of several genes involved in sphingolipids (Zhu 2008) or storage protein synthesis (Lara-Chavez et al. 2012) in *P. taeda*, as well as triacylglycerol and/or storage protein contents in *P. taeda* (Brownfield et al. 2007) and *P. strobus* (Klimaszewska et al. 2004). ABA present at increased concentrations during *se* maturation in loblolly pine affected their gene expression profile, which was more similar to that of mature *ze* (Vales et al. 2007).

Cotyledonary *ze* undergo many changes during the late maturation and post-maturation stages. Water loss is accompanied by a sudden increase in protein content (over 50 % per mg DW), the synthesis of storage proteins (vicilin- like proteins, legumin-like proteins, albumin-3 and pine globulin-1) and a decrease in sugar content (by 40 % per mg DW) (Morel 2014). Additionally, a change in the type of sugars is observed, namely the disappearance of hexoses and an increase in the raffinose family of oligosaccharides (RFO) (Pullman and Buchanan 2008; Morel et al. 2014b), which are known to be involved in the acquisition of desiccation tolerance (Lipavská and Konrádová 2004). It may be important to stimulate those biochemical changes as well as post-maturation treatment (Maruyama and Hosoi 2012) in *se* through the use of adequate tissue culture conditions to ensure high germination capacity and conversion rates into plants.

19.6 Implementation of SE for Tree Breeding and Forest Regeneration

According to a survey done in 2007, pines are among the forest tree species for which the use of vegetative propagation (VP) through rooted cuttings is wide spread, with approximately 164 million plants produced annually worldwide (Lelu-Walter et al. 2013). For the production of these materials, SE, either alone or in combination with organogenesis and rooted cutting propagation, are used. Likewise, VP including SE can facilitate both tree breeding (greater selection accuracy and gains, breeding archives of donor material for making crosses after the selection) and implementation of deployment strategies for improved reforestation materials.

19.6.1 Integration into Breeding Programmes

The overall scheme of implementation of SE for tree breeding and for the production of improved forest regeneration material is shown in Fig. 19.10, and has

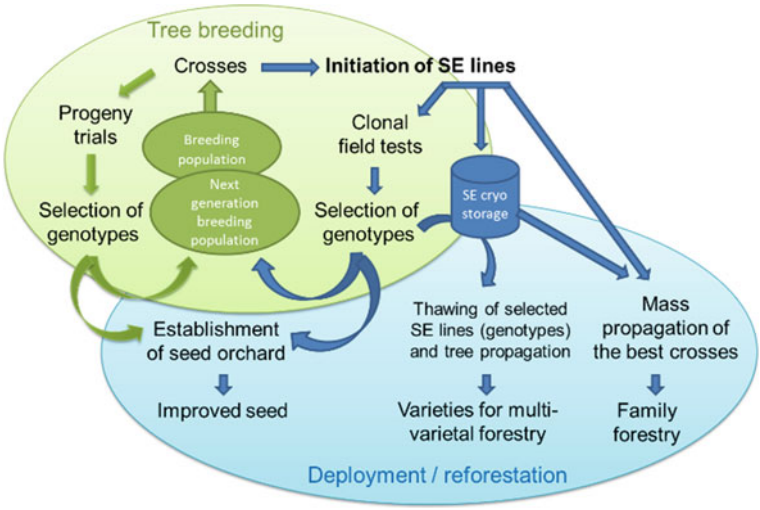

Fig. 19.10 Diagram of the implementation of somatic embryogenesis (SE) in *Pinus* tree breeding and in the production of improved forest regeneration material

recently been reviewed by Krakau et al. (2013) and Lelu-Walter et al. (2013). A description of SE applications in pine improvement and deployment strategies in plantation forestry can also be found in Klimaszewska et al. (2007). In short, VP (e.g. by means of SE) provides several advantages for breeding through clonally replicated genetic tests (clonal testing for forward selection): when multiple copies of the candidates to be tested can be placed in various environments, microenvironmental and genotype by environment interactions as well as phenotypic plasticity can be estimated more accurately than by progeny testing. As a result, both genetic parameter estimation and selection accuracy are improved following clonal testing. Shortening of breeding cycles is another remarkable benefit achieved by clonal testing when compared with progeny testing in which extra time is needed to allow candidates to flower prior to testing their progenies. In *P. sylvestris*, the cycle length was estimated to be 33–36 years when using different breeding strategies based on a separate progeny testing step, and only 23 years when selection is performed in field tests with cloned replicates rather than seedlings (Rosvall and Mullin 2013). VP is also useful at the time of genetic testing for varietal selection prior to deployment. In such varietal tests, a number of selected families, varieties per families (genotype/progeny) and ramets per variety are field tested for accurate selection of tree varieties that will be used in plantation forestry (Weng et al. 2011). In practice, varietal tests can be advantageously set up to maximize genetic gains at a genetic diversity level above a fixed threshold of acceptability.

SE is also well suited for early selection schemes, in which molecular markers for specific traits (maker-assisted selection/MAS) or genomewide information

(genomic selection/GS) is used as the basis for selection. For example, in *P. taeda,* genomic selection is expected to lead to 53–112 % higher selection efficiency per time/unit, i.e. a reduction of 50 % in the breeding cycle (Resende et al. 2012). Integration of both MAS or GS selection and SE into conventional breeding programmes is likely to result in high synergies over the next decades for the implementation of multi-varietal forestry (El-Kassaby and Klápště 2015; Plomion et al. 2016). Defined as the use of tested tree varieties in plantation forestry, multi-varietal forestry is an efficient way of preserving productivity and adaptability of intensively managed conifer plantations using all available genetic variance, i.e. both additive and non-additive genetic variances (Park 2002; Weng et al. 2011).

However, it is well known that there is variation among families in the success of SE, i.e. it can be difficult to obtain SE lines from all the elite crosses, and the potential impacts of low or variable genotype capture on the outcomes of the breeding have raised some concerns. However, a simulation study by Lstibůrek et al. (2006) showed that variation in the success of clonal propagation does not lead to reduced genetic gains in the selected clonal mixtures since most of the variation in the breeding population is within-family variation, and superior clones may thus originate from other crosses of the very best parents. It should be noted that significant progress has been made in improving genotype capture at the initiation step from crosses of interest in *P. pinaster* and *P. radiata* in recent years (see above *Induction of somatic embryogenesis* in *Pinus* spp.). As a result, the prospects for incorporation of SE technology into broader forestry programmes are good (Lelu-Walter et al. 2006; Hargreaves et al. 2009, 2011; Trontin et al. 2016a).

In practice, clonal testing is seen as complementary to progeny testing, and tree breeding programmes combine the two to achieve the best selection accuracy (Baltunis et al. 2009). For example, a new breeding strategy adopted by the Radiata Pine Breeding Company for New Zealand and New South Wales, Australia, includes an elite population tested both as progeny and as clones (Dungey et al. 2009). The idea is that clonal populations will capture the greatest gains in traits having low heritability, and the progeny will make it possible to test more families and individuals for traits with higher heritability. The presence of clones will also allow destructive testing for selection criteria such as wood quality characteristics. The elite population is managed intensively following a "rolling front" approach, i.e. new material is being created and testing is conducted continuously. At the same time, pre-deployment selections from the best parents are done for the development of commercial clonal varieties. In *P. sylvestris*, Rosvall and Mullin (2013) showed through computer simulations of alternative breeding strategies that the genetic gain per time unit depends on the selection intensity and accuracy and also on the time required to complete the breeding cycle. A breeding strategy based on clonal testing will have a moderate selection intensity (because of the high resources needed to produce clonal ramets for each tested tree), but a high selection accuracy, a short cycle time and a high potential for genetic gains. Conversely, strategies based on progeny testing can offer higher selection intensity, but the breeding cycle time is longer and the potential for genetic gains per year is therefore reduced.

Total cost per breeding population and breeding cycle for a strategy based on clonal replicates (short cycle time) has been estimated at € 233 k in *P. sylvestris* (Rosvall and Mullin 2013). This is slightly higher than the costs estimated for other strategies based on progeny testing and with long cycle time (€ 204–218 k) and can mainly be explained by the need for the production of propagules. SE may help to reduce the cost of producing clonal propagules since it is potentially a very efficient multiplication technology.

19.6.2 Options for Deployment

Traditionally, in pines, improved forest regeneration materials are produced in seed orchards in which the selected trees are clonally propagated, often by grafting, and mate to produce seeds. If the planting stock for reforestation can be vegetatively propagated, e.g. through SE, several benefits are achieved (Klimaszewska et al. 2007; Dungey et al. 2009; Lelu-Walter et al. 2013). Improved material will be available for production forestry faster since there is no delay due to the time needed for seed orchards to grow and start flowering. Likewise, irregular flowering or pests and pathogens affecting seed yields do not cause uncertainty in the supply of improved material. In VP, the genetic gains achieved by breeding are transferred as a whole (both additive and non-additive variation) to production forestry. It is also possible to maximize genetic gains by implementing multi-varietal forestry, i.e. by focusing on the multiplication of the very best individuals. This is currently being demonstrated with *P. radiata*. In parallel with optimizing varietal production methods, Forest Genetics (www.forest-genetics.com) has been actively developing an advanced generation of improved varieties. This was done by creating new varieties from crosses among the best clones already in production (Carson et al. 2015).

Deployment through VP provides an opportunity to select clones for specific end uses or specific site conditions. The material to be propagated can also consist of hybrids. SE is compatible with the propagation of pine hybrids. A good example of this is the ongoing work conducted with *P. attenuata* x *P. radiata* hybrid SE lines in New Zealand, aiming to improve performance under dry and cold growth conditions that are not well suited for either of the parent species (Anonymous 2015). SE methodologies may be of special benefit when using hybrid pines to facilitate the capture of EM before any incompatibility issues with the crossing of different species may arise that could result in empty seeds at maturity.

With VP, the growing stock will be more uniform, which could reduce harvesting and processing costs; also, genetic diversity may be reduced. This may increase the risk for disease or pest damage if susceptible clones are widely used, and some concern has also been raised on clonal stability over various environments (Bettinger et al. 2009). However, recent results, (e.g. from *P. radiata*)

suggest that clones performing well and showing stability over large areas can be found (Baltunis and Brawner 2010). Moreover, genetic diversity can be maintained by establishing polyclonal plantations as well as mosaics of species and genotypes at the landscape level, and by the continuous release of new and better clones (Bettinger et al. 2009; Carson et al. 2015). Multi-varietal forestry can be advantageously used to guarantee that the genetic diversity of a varietal mixture is fixed to a given acceptable level and that genetic gain is maximized under this constraint (Weng et al. 2011 and reference therein).

Options for the deployment of SE (or other types of VP) are presented in Fig. 19.10: SE can be utilized for the mass production of tested and selected clones that are marketed either as single varieties or as varietal mixtures. Alternatively, the best seed families can be multiplied without clonal testing, resulting in family forestry (Dungey et al. 2009; Lindgren 2009; Krakau et al. 2013; Lelu-Walter et al. 2013). Yet another option is to convert clonal trials into varietal tests and, ultimately, into seed orchards after testing for and removing of inferior clones (Dungey et al. 2009), followed by traditional seed production. In such a scenario, testing efforts should be carefully estimated using computer simulations to optimize the costs of establishment of clonal trials, varietal tests, and/or seed orchards (Weng et al. 2011).

When SE is used for mass propagation, *ss* can be either directly used as planting stock, or they can be grown as cutting donors in nursery stool beds or used as stored embryogenic cultures. In *P. taeda* (Soresson 2006; Bettinger et al. 2009), in which rooting of shoot cuttings is difficult, direct planting of germinated *se* cannot be used. In *P. radiata*, production of rooted cuttings from plants lined out in the nursery bed works well, which results in cheaper planting stock than that obtained with the direct use of *ss* (Carson et al. 2015). Several further improvements have been made to integrate *P. radiata* SE technology into less intensive propagation methods. Germinated *se* can be used to provide shoots for the initiation of organogenic cultures, which is done by trimming the root and hypocotyl from *se* and placing them on standard multiplication medium (details on transfer strategies and media are provided in Hargreaves et al. 2005; Hargreaves and Menzies 2007). Plantlets produced from organic cultures derived from *se* are shown in Fig. 19.11. Other approaches include using the first plants that arise from *se* as stool plants, that can be intensively managed in pots and propagation houses (where small needle fascicle cuttings can be taken multiple times throughout the growing season) or lined out in nursery beds as described earlier. Some attempts have been made to use *se* tissue (cotyledons and epicotyl material) to regenerate shoots via adventitious meristem formation (Montalbán et al. 2011). However, research by Hargreaves et al. (2005) indicates that the process of adventitious shoot formation may confer a legacy of increased maturation, which results in slower in vitro and ex vitro growth and poorer root initiation. Phenotypic differences in bud development were also observed after 18 months in the nursery (Hargreaves et al. 2005).

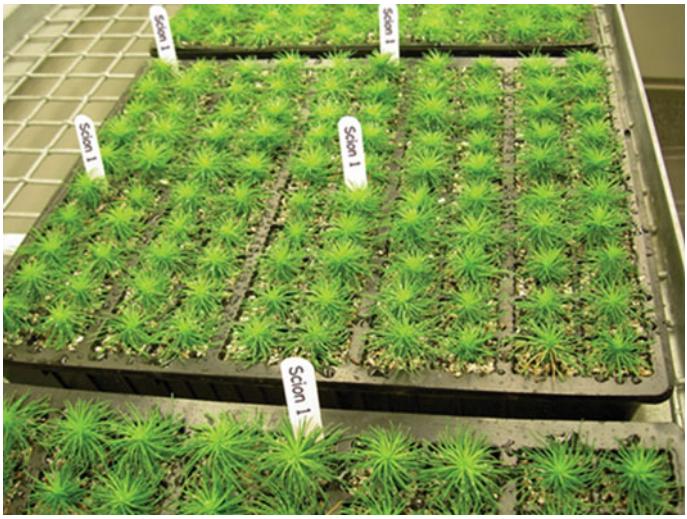

Fig. 19.11 Organogenic shoots of *Pinus radiata* generated at Scion, New Zealand, from germinated somatic embryos (root initiation phase)

19.6.3 Field Performance of SE-Derived Pines

The predicted gains associated with multi-varietal forestry, i.e. the use of vegetatively propagated planting stock instead of seedlings, are high: productivity of the selected elite clones is expected to increase by up to 50 %, with potential simultaneous improvements in stem and wood quality and tree resistance (Soresson 2006). Since the application of SE to pine species is still a relatively novel approach, the data on actual field performance of pine *ss* is still scarce, although it is accumulating all the time.

A recent study by Antony et al. (2014) in the Southern US compared the growth and wood properties of *P. taeda ss* with full-sib and half-sib seedlings after four growing seasons in the field. They found that overall growth properties were better in full-sib seedlings than in *ss*, but *ss* had higher wood density. Furthermore, it was possible to find clones showing both better growth and higher wood density, two traits that are generally negatively correlated. Wood et al. (2015) examined an 8-year-old field trial of *P. taeda* established with *ss* and concluded that selecting clones based on total height and stem diameter will result in varieties with the largest volume growth, even if stem taper is not taken into account.

In *P. radiata*, assessment of the stem diameter of 5-year-old *ss* and 4- to 13-year-old seedlings showed that the deployment of the top 5 % of clones would improve more than 100 % of the family forestry (seedlings from controlled crosses of the selected parents) at the same selection intensity (Baltunis et al. 2009). As in *P. taeda*, "correlation breakers", i.e. clones having both improved growth and wood quality can be found in *P. radiata* (Cown and Sorensson 2008). The recent

assessment of 9-year-old *P. radiata* trials has revealed *se*-derived clones having 11–21 % better height growth and 5–14 % larger stem diameter and, at the same time, remarkably improved wood quality (+8–17 % wood density, +38 % core-wood stiffness) when compared with seedlings of either seed orchard or controlled crosses origin. An important part of field evaluation of *se*-derived *P. radiata* clones in New Zealand included selection for resistance against the fungal disease *Dothistroma* needle blight, which can have a significant impact on early tree growth (Carson et al. 2015).

In France, eight field trials have been established with *P. pinaster ss* and control seedlings in a nursery (since 1999) or in the forest (since 2004) (Trontin et al. 2016a). After 15 years of growth, the oldest test demonstrated that *ss* had completed all development phases, from juvenile growth to the adult vegetative and reproductive phases (flowering and production of cones). In both nursery and forest plots, the mean initial height of somatic tree clones was lower than that of control seedlings of the same or improved families, and a similar conclusion was obtained for most clones at age 6–7 years. Such a poor initial growth performance of *ss* in *P. pinaster* was recently associated with inappropriate biological, physiological and molecular characteristics of cotyledonary *se* harvested after 12 weeks of maturation as compared with the quality standard of seedlings (Morel et al. 2014b, see *Molecular aspects of SE and ZE in pine* in this chapter). However, growth rates of somatic clones, computed as the mean relative increase in height 6–7 years after plantation, were reported to be either similar or better than those of control seedlings (Trontin et al. 2016a). It was concluded that somatic clones could recover from initial low vigour after a few years under field conditions and subsequently perform as seedlings. Selecting the top ranking clones within each family may therefore already provide opportunities for improved traits. However, further refinement of maturation conditions to improve the quality of cotyledonary *se* is necessary to achieve full genetic gains with varietal mixtures of somatic clones.

Behaviour of *P. sylvestris ss* under the field conditions in comparison with seedlings of the same genetic background was recently studied in Finland by Aronen (2016). At planting time, *ss* were smaller than the seedlings, but they grew normally. During the first six growing seasons, their annual height growth was comparable or, in the case of some clones, inferior to that of the seedlings. However, the number of clones studied was small, so no definite conclusion could be made. There are currently no large-scale efforts to use SE in breeding or reforestation of *P. sylvestris*, but the *ss*-derived clones having variable contents of phenolics are being tested for their fungal resistance both in the field and under controlled conditions.

The propagation methods used for *ss*-derived plants of *P. radiata* in New Zealand have gone a long way towards reducing earlier differences in field performance observed in other *Pinus* species described in this section. A significant proportion of the planting stock in New Zealand is derived from seedling cuttings of control-pollinated crosses (Hargreaves et al. 2011). The use of either *se* or shoots derived from organogenic cultures to subsequently form stool beds means that the production methods are identical; cuttings are sturdy with good fibrous root systems.

19.6.4 Regulations and Other Factors Affecting Implementation

Implementation of SE into practical forestry is advancing in pine, but it also faces to same challenges as any breeding programme, including biological (target species, ecological conditions), economic (value of target traits, return on investment, operating funds), institutional (cooperation between academic, private and government organizations) and sociopolitical (social expectations, legal requirements) factors (Rosvall and Mullin 2013). Globally, there are no specific rules governing the use of vegetatively propagated reforestation materials. In the European Union, however, many member states have rather strict regulations concerning testing requirements, the maximum number of plants per clone, the minimum clone number, the maximum percentage of area where clonal materials are allowed in forestry and so on. Another obstacle is that none of the current forest certification standards approve vegetatively propagated materials (Bettinger et al. 2009; Lelu-Walter et al. 2013).

Also, the lack of public acceptance and lack of interest on the part of forest owners to pay more for improved planting stock may hinder the application of VP in forestry. However, as with regulations, these issues vary a lot depending on the region and the species. For SE, development of cost-efficient mass propagation technology is a key issue, especially for the species for which production costs cannot be lowered by further propagation via rooted cuttings. The price for *P. taeda ss* is about six times the price of a seedling, and even if the expected economical outcome at the final harvest is substantially better with clonal material than when using seedlings (Bettinger et al. 2009), establishment costs may still be too high for some of the landowners. The SE costs can be reduced by lowering planting densities and by mixing clonal plants with seedlings (Bettinger et al. 2009; Lelu-Walter et al. 2013), but eventually, the development of automated technology for *ss* production will be needed. This work is currently ongoing and either published (Aidun and Egertsdotter 2012; Anonymous 2014) or patented, e.g. in the case of *P. taeda* by the Weyerhaeuser Company with various published patents, in particular in the field of manufactured seed construction (see Gupta et al. 2014 for patent numbers).

19.7 Conclusions and Future Research

Somatic embryogenesis coupled with long-term storage of EM genotypes at low temperature is a modern and powerful tool for scaling up the production of genetically improved conifer varieties and storage of genetic resources. Protocols for both SE and cryopreservation of embryogenic tissues are being developed for an increasing number of conifer species including pines (Lelu-Walter et al. 2013; Klimaszewska et al. 2016). In contrast, achieving SE from explants of mature trees is still challenging (Klimaszewska and Rutledge 2016; Trontin et al. 2016c) but

remains to be a highly strategic research field because direct propagation of elite trees with field-proven performance would result in a significantly shorter breeding cycle and overall reduced costs to deliver new varieties in plantation forestry. In New Zealand, two companies are commercially producing planting stock derived from the SE process (Forest Genetics and Arborgen). Plants are produced via a combination of propagation techniques using *se* directly as stock plants for fascicle cuttings or incorporating an in vitro organogenesis step to provide plants that are used as planting stock or for stool bed production (then cuttings). The laboratory methodologies are still relatively expensive and SE-derived planting stocks cost more to produce; any progress towards more efficient or possibly automated technologies is essential.

In addition to cell culture improvements, molecular studies have been undertaken in several economically important pine species (mainly *P. pinaster*, *P. radiata*, *P. sylvestris* and *P. taeda*). These results have increased our knowledge of genes and molecular pathways involved in (i) embryogenecity (determined by SE initiation rate), (ii) the notorious loss of embryogenic competence (based on maturation capacity) in ageing cultures and (iii) inferior embryo quality (as estimated by comparison with *ze*: lower *se* germination capacity and lower initial growth rate of *ss*). A number of molecular tools are already available for use to discover and study the effect of genetic instability on SE initiation frequency, *se* maturation yield and embryo quality. However, the biological significance of observed variations in pine and other conifers is still questionable and would require more functional studies (Miguel et al. 2016). New knowledge about the expression of gene cohorts involved in normal embryo patterning, from early embryo proliferating in EM to late embryo cotyledonary stages, has been gathered from targeted gene and transcriptomic or proteomic profiling in pines and other conifers (Trontin et al. 2016b). In pines, there is already strong evidence in pine of stage-specific modulation of embryogenesis-related genes by a number of transcription factors and epigenetic mechanisms (Oh et al. 2008; Klimaszewska et al. 2009; de Vega-Bartol et al. 2013; Morel et al. 2014a).

A number of genes and associated pathways have emerged as excellent candidates to provide molecular tools of practical interest to refine SE at the maturation step but they require confirmation studies in different pine species (Miguel et al. 2016). Comparative biochemical, transcriptomic and proteomic analysis of SE and ZE can be used to gain a better understanding of *se* quality. Careful examination of the molecular data from these comparative studies may prove essential for refining maturation or post-maturation protocols in pines.

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