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Vegetative propagation of larch species: somatic embryogenesis improvement towards its integration in breeding programs

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Abstract

Vegetative propagation of forest trees offers advantages to both tree breeders and the forest industry. In larch vegetative propagation has chronologically followed three major developments: (i) clonal propagation by cuttings; (ii) 'bulk' vegetative propagation by cuttings and (iii) somatic embryogenesis. The latter has potentially numerous applications such as the production of a large number of genetically improved plants and the amenability of embryogenic cultures to be stored in liquid nitrogen. In *Larix* sp. several improvements of the somatic embryogenesis protocol have been developed. Maturation conditions are now well enough refined to regenerate high quality somatic embryos that are highly similar to zygotic embryos in their anatomy, physiology and protein content. Among conifer species, somatic embryogenesis of *Larix* has become a model for its multiple uses; its integration in a breeding program is now undertaken for clonal propagation of improved material of hybrid larch *Larix* x *eurolepis*. Indeed, somatic embryogenesis assists breeding strategies by offering an alternative tool for at the same time accelerated production of plants for clonal testing and then for mass production; in addition, cryoconservation allows keeping material in a juvenile state and allows mass production of stored material at any time. This review describes the different methods of vegetative propagation of larch sp., in particular the advances in somatic embryogenesis and requirements for its integration into a breeding program.

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Keywords: Breeding; *Larix;* marker; maturation; embryo quality; protein content; zygotic embryo.

1. **Larch species**

The genus *Larix* is attractive to reforestation programs due to its fast growth, wide ecological plasticity and good wood quality (Gower and Richards 1990). Larch (*Larix* sp.) is one of the major components of coniferous forest in the Northern Hemisphere, where it is represented by around 10 species. In Europe, foresters are interested in the local species European larch (EL, *L. decidua*) -mostly growing in mountainous areas (mainly in the Alps, Tatras, Sudeten Mts)- and by Japanese larch (JP, *L. kaempferi*), a species from high elevation mountains in Honshu-Japan. Other species are also of interest in Northern Scandinavia where Sukaczewi and Siberian larches grow better. Due to their fast juvenile growth, fine architecture and wood properties (mechanical strength and durability), foresters have since long attempted to plant larches well-beyond their native range, in France, Germany and Poland in lowlands, but also in Northern and Western Europe. Success is mitigated. Failure of EL in Western Europe due to larch canker stressed the need for the proper choice of origins and this stimulated provenance research (IUFRO trials). Successful Japanese larch plantations were restricted to oceanic coastal areas where they do not suffer from summer drought. The recent explosion of *Phytophtora ramorum* in the UK and in Ireland in plantations of the very sensitive JL restricts from further plantation expansion (Webber et al. 2010). Problems due to canker (*Lachnellulla willkommii*) on EL (especially from alpine origins) plantations in France have recently raised concerns (Piou et al. 2013). A third larch taxa of prominent interest in Europe but also in North America is the interspecific hybrid between European and Japanese larches (*L.* x *eurolepis*). It is the correspondent in Eastern Asia of the *L. kaempferi* x *L. gmelini* hybrid. Discovered because spontaneous crossings had occurred at Dunkeld, Scotland at the beginning of the 20th century, *L.* x *eurolepis* has quickly attracted foresters but also breeders for its fast growth and benefits gained from complementary traits. Heterosis has often been advocated as an explanation for this superiority over its pure parents but it has only recently been demonstrated that this is in fact correct (Pâques et al. 2013). Danes created their first hybridization orchards in the nineteen forties (some are still producing commercial crops); they were followed by nearly all other European countries that established new seed orchards. The main drawback of open-pollination interspecific hybridization orchards is their unpredictable and changing rate of hybrid seed production from orchard to orchard and even from year to year. Recent results have shown hybrid rates from less than 20% to up to nearly 80% (Pâques et al. 2006). Mismatching of flower phenology

between EL and JL is the main cause. To overcome this instability, separate seed orchards for EL and JL have been established in France and supplemental pollination is used. High hybrid rates are observed (over 90-95%) but because of the extra cost for artificial pollination and of the actual low seed yield in larch compared to other *Pinaceae*, hybrid larch seed is produced at too high a cost. This seriously impedes hybrid larch deployment in plantation.

2. Vegetative propagation

Besides generative reproduction, vegetative mass-production of hybrid plants was attempted to circumvent weaknesses in orchard seed production: vegetative propagation maintains a high level of hybrid purity and attains stabilization of hybrid varieties, higher genetic gains, and better uniformity of crops. Vegetative propagation of larch has followed chronologically three major developments (Pâques et al. 2013): (i) clonal propagation by cuttings; (ii) 'bulk' vegetative propagation by cuttings and (iii) somatic embryogenesis (Figure 1).

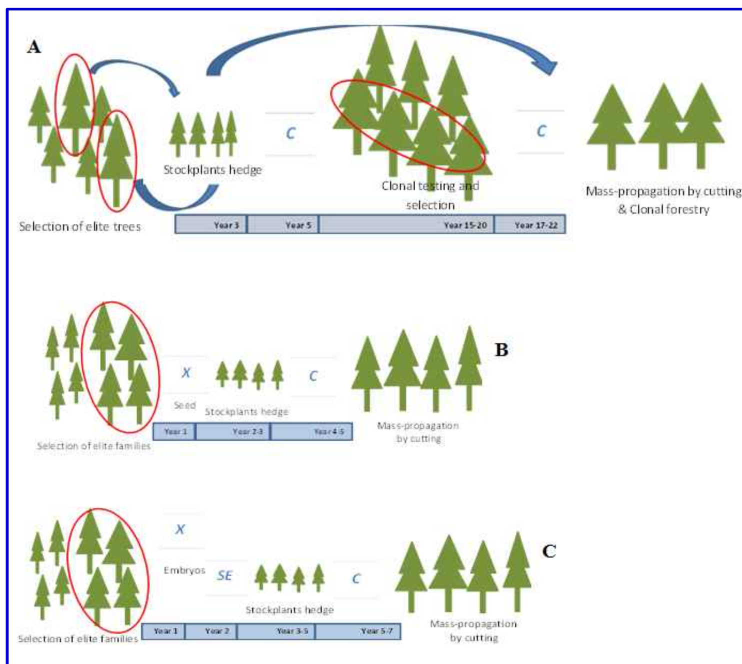

Figure 1. *Hybrid larch vegetative propagation scenarios:* ***A)*** *'clonal' propagation by cuttings,* ***B)*** *'bulk' propagation by cuttings and* ***C)*** *combined somatic embryogenesis and 'bulk' propagation by cuttings. C = cuttings; X = control crossing; SE = somatic embryogenesis*

Clonal propagation by cuttings followed the scenario developed for Norway spruce by Kleinschmit in Germany (Kleinschmit et al. 1973), with its different steps:

selection of young seedlings the in nursery; plantation of ortets in dedicated stockplant banks with appropriate cultivation management of trees; selection of best clones in clonal trials and finally mass-propagation for commercial plantation (clonal forestry). The rapid ageing of stockplants -whatever the technical attempts to maintain juvenility- resulting in low rooting rate and plant plagiotropism led to its commercial failure. Ageing of stockplants and its related problems were circumvented by the so-called 'bulk' propagation by cuttings (Pichon et al. 2001). In this scenario, young seedlings from selected hybrid full-sib families (produced by controlled crossings) are raised as stockplants to feed annual needs of cuttings up to the age where they become physiologically too old for proper rooting (3-4 years). Stockplants are thus regularly renewed over time. Successful results have been obtained in France up to the level of pilot-scale experimental production with nice growing plantations. But because of their higher cost (1.5-2 times that of sexually produced plants), rooted cuttings could hardly compete with seedlings in our context, and the transfer of this technique to commercial private nurseries has not been successful so far. In another forestry and economical context (namely in Québec), several hundreds of thousands of such cuttings are produced yearly and deployed in the forest [https://www.mffp.gouv.qc.ca/forets/semences/semences-production-techniques-resineuses-boutures.jsp]. A third alternative for vegetative propagation is through somatic embryogenesis.

3. Somatic embryogenesis in larch species

Somatic embryogenesis has become a method of choice for clonal propagation of forest trees, due to its high multiplication rate and the amenability of embryogenic cultures to cryogenic storage. This biotechnology constitutes a tool for rapid propagation of material from breeding programs (Lelu-Walter et al. 2013). Advances in conifer somatic embryogenesis in the last 10 years have been reviewed recently (Klimaszewska et al. 2015). In *Larix* somatic embryogenesis has been achieved in several species (Bonga et al. 1995). It was first reported in by Klimaszewska (1989a) for hybrid larches *Larix* x *eurolepis* Henry (*L. decidua* x *L. kaempferi*) and *Larix* x *marschlinsii* Coaz (*L. kaempferi* x *L. decidua*). Since then it has been obtained for European larch (*L. decidua* Mill.) (Cornu and Geoffrion 1990; von Aderkas et al. 1990; Szczygieł 2005), Japanese larch (*L. kaempferi* Gond.) (von Aderkas et al. 1990; Kim et al. 1999), western larch (*L. occidentalis* Nutt.) (Thompson and von Aderkas 1992), eastern larch (*L. laricina*, (Du Roi) K. Koch tamarack) (Klimaszewska et al. 1997) and more recently for Siberian larch (*L. sibirica*) (Tretiakova et al. 2012). Significant improvements in the somatic embryogenesis process have been obtained for Japanese larch (Kim and Moon 2007; Zhang et al. 2010) and hybrid larches (Lelu-Walter and Pâques 2009).

4. Somatic embryogenesis and other biotechnologies

Conifer somatic embryogenesis has potentially numerous applications. Indeed, this efficient method of plant regeneration constitutes a tool for research (study of gene function, reviewed by Trontin et al. (2015)) and for species improvement (production of a large number of genetically improved plants). Embryonal cultures (named embryonal masses in conifers) constitute an interesting material which can be used for other applications than plant regeneration (Figure 2). Efficient protocols have been developed to cryopreserve embryonal masses in liquid nitrogen. Cryopreservation offers real new perspectives for long-term conservation of the embryonal masses without loss of juvenility and their reactivation at any time (Park *et al.* 1998).

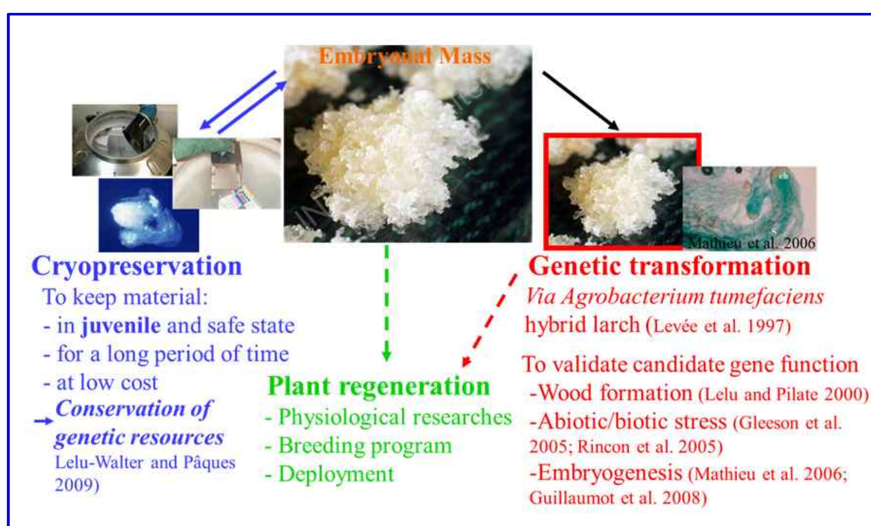

Figure 2. *Somatic embryogenesis of hybrid larches (Larix x eurolepis, L. x marschlinsii): for fundamental and applied research.*

Improvement of tree species using conventional plant breeding techniques is a long process and genetic transformation may help to speed it up. Genetic transformation was first obtained using a microprojectile bombardment protocol but first attempts in *Larix* sp. gave only rise to transient expression (Duchesne et al. 1993); subsequently transgenic tamaracks (*Larix laricina*) were regenerated but the transformation efficiency remained low (Klimaszewska et al. 1997). Transformation via *Agrobacterium tumefaciens* has been successfully achieved for the first time for hybrid larch (*Larix* x *marschlinsii*) giving rise to stable genetic transformation followed by plant regeneration (Levée et al. 1997). The improved procedure gave rise to high transformation rates, an average 108 ± 5.7 (mean ± 95% CI) and 154 ± 19.6 (mean ± 95% CI) resistant embryonal masses per gram fresh

weight (i.e. 15-16 transformation events per Petri dish) for SCOMT (Caffeic acid O-methyltransferase) and SCCR (Cinnamoyl Coenzyme A Reductase) respectively (Lelu and Pilate 2000). In hybrid larch, this efficient genetic transformation has been used to study gene function involved in wood formation (Lelu and Pilate 2000), in abiotic and biotic stress (Gleeson et al. 2005; Rincon et al. 2005) and more recently in embryogenesis (Mathieu et al. 2006; Guillaumot et al. 2008). Finally we have to mention that embryonal masses have been used as a source of protoplasts (Korlach and Zoglauer 1995) in order to recover somatic embryos and plants as was successfully demonstrated for *Larix* x *eurolepis* (Klimaszeswka 1989b). The ultimate purpose was the fusion of protoplasts to produce hybrids with novel genetic combinations (Pattanavibool et al. 1998). Also of interest is the fact that *Larix decidua* plants have been regenerated from haploid megagametophyte tissue *in vitro* (see von Aderkas et al. in this book).

5. Technical aspects of somatic embryogenesis in larch sp.

5.1 Improvements of the different steps

Recovery of embryonal masses depends on the developmental stage of the zygotic embryo. Research carried out over the last two decades has proven that somatic embryogenesis is initiated most efficiently from immature zygotic embryos (Table 1). Indeed, there has been limited progress towards initiating

***Table 1**. Induction of somatic embryogenesis in the Larix sp.*

Species	Explant type	Initiation (% max)	References
Larix decidua	Precotyledonay ZE	21	von Aderkas et al. 1990
	Immature ZE	36	Szczygieł K et al. 2007
	Mature ZE (stored seed)	5	Lelu et al. 1994c
Larix kaempferi	Precotyledonay ZE	17	von Aderkas et al. 1990
		67	Kim *et al.* 1999
Larix laricina	Precotyledonay ZE	44	Klimaszewska et al. 1997
Larix occidentalis	Immature coty. ZE	93	Thompson and von Aderkas 1992
Larix sibirica	Immature coty. ZE	18	Tretiakova et al. 2012
Larix x *eurolepis* (*L. decidua* x *L. kaempferi*)	Precotyledonay ZE	15	Klimaszewska 1989a
		78	Lelu-Walter and Pâques 2009
Larix x *marschlinsii* (*L. kaempferi* x *L. decidua*)	Precotyledonay ZE	25	Klimaszewska 1989a
	Precotyledonay ZE	62	Lelu et al. 1994c
	Immature coty. ZE	26	Lelu et al. 1994c
	Cotyledonary SE	98	Saly et al. 2002
	Needle (embling)	3	Lelu et al. 1994c

SE: somatic embryo; ZE: zygotic embryo ; emblings: plant regenerated from a somatic embryo.

somatic embryogenesis from mature seeds. For the hybrid, *Larix* x *marschlinsii*, needles from somatic plantlets (emblings) yielded embryonal masses at a lower frequency (3%) than did mature somatic embryos of the same genotype (83%, Lelu *et al.* 1994c). This decrease in embryogenic ability of the explants could be attributed to differentiation related to maturity of the explant (von Aderkas and Bonga 2000). So far, somatic embryogenesis from mature larch trees has not been obtained and remains a challenge despite attempts giving rise to embryo-like structures (Bonga 1996; 1997).

Once obtained, embryonal masses are transferred onto maintenance medium and sub-cultured every 2 weeks onto fresh medium in order to sustain their proliferation. Embryonal masses can be stored in liquid nitrogen (-196ºC). For *Larix* x *eurolepis* a cryopreservation method was first developed using a programmable freezer (Klimaszewska et al. 1992). Embryonal masses were pre-grown for 24h in medium with 0.4M sorbitol, treated with DMSO 10% before controlled cooling to -40°C. The vials were then submerged and stored in liquid nitrogen. A simplified cryopreservation method (no need for a programmable freezer) was subsequently developed (Lelu-Walter and Pâques 2009). Embryonal masses pre-grown for 24h in medium with 0.4M sucrose, treated with DMSO 10% were placed in a freezer at –80°C for 2 h (in Nalgene™ Cryo 1°C Freezing Containers). The vials were then stored in liquid nitrogen. Cryopreservation techniques have resulted in the recovery of all tested lines. The cryopreservation *per se* and its duration (at least up to 18 years) had no apparent effect on the yield of somatic embryos (Lelu-Walter and Pâques 2009).

For larch sp., as for other conifers, somatic embryo development was improved in the presence of abscisic acid (ABA), its concentration varying according to the species with 40-60µM being the most common concentration range used (Table 2). This resulted in synchronous development of cotyledonary somatic embryos without precocious germination (Lelu and Label 1994). ABA influenced tissue differentiation in larch (Gutmann et al. 1996) and promoted storage products such as lipid and protein accumulation in embryos (Gutmann et al. 1996; von Aderkas et al. 2002). The osmolarity of the culture medium is another important factor. In general the sugar content, either sucrose or maltose is increased (0.2M-0.4M, Table 2); for *L. laricina* and *L. sibirica* polyethylene glycol (PEG) has been added to the maturation medium (Klimaszewska et al. 1997; Tretiakova et al. 2012 respectively). More recently, somatic embryo development has been improved under reduced water availability, i.e. by a high gellan gum concentration, up to 0.8%, for both Japanese (Kim and Moon 2007) and hybrid larches, *Larix* x *eurolepis* and *Larix* x *marschlinsii* (Lelu-Walter and Pâques 2009). For the latter, the number of somatic embryos produced among the embryogenic lines tested (23) is ranging from 8 to over 1500 per g fresh weight of tissue (Lelu-Walter and

Pâques 2009), a phenomenon commonly observed with conifer species. When using the improved protocol for hybrid larch, 94% of the lines produced mature somatic embryos (Figure 3). Recovery of high quality somatic embryos resulted in high germination and plant formation frequencies (96 and 65% respectively, Lelu-Walter and Pâques 2009).

***Table 2**. Somatic embryo maturation in the Larix sp: optimal conditions*

Species	ABA (μM)	Sugar (M) / PEG (g L^{-1}) *	Gellan gum (g)	Embryogenic Pot** N° coty SE/ g F W (*N° line tested*)	References
Larix decidua	60	Suc 0.2	4	226 (*6*)	Szczygieł K et al. 2007
Larix kaempferi	60	Mal 0.2	8	392 (*1*)	Kim and Moon 2007
Larix laricina	40	Suc 0.4/50	4	316 (*1*)	Klimaszewska et al. 1997
Larix occidentalis	0.025	nd	4	30 (*7*)	Thompson and von Aderkas 1992
Larix sibirica	121	Suc 0.1/100	4	400 (*7*)	Tretiakova et al. 2012
Larix x *eurolepis*	60	Suc 0.2	8	1566 (*23*)	Lelu-Walter and Pâques 2009
Larix x *marschlinsii*	60	Suc 0.2	4	403(*6*)	Lelu et al. 1994a
	60	Suc 0.2	8	2430 (*1*)	Lelu-Walter and Pâques 2009

* Suc : sucrose ; Mal : maltose; PEG: polyethylene glycol; ** Highest response. nd: non disponible.

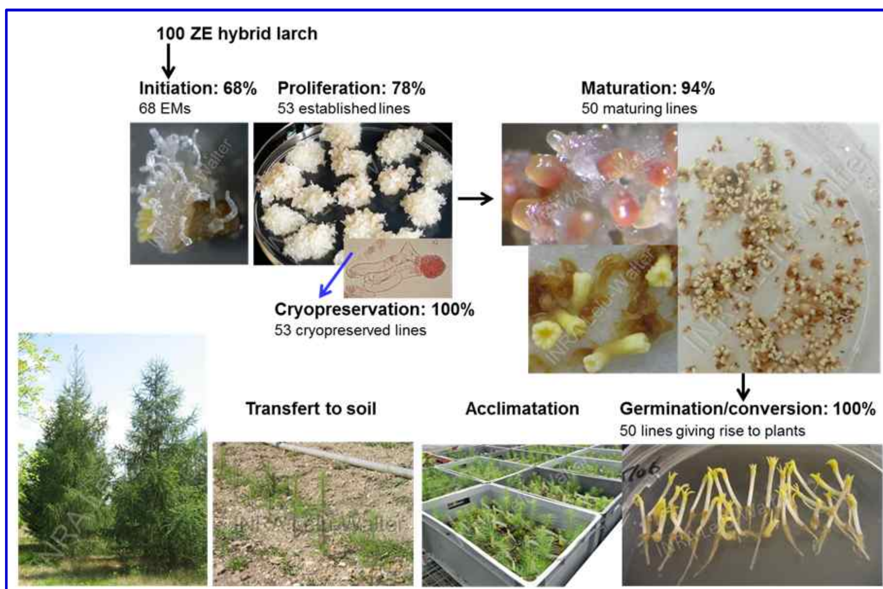

***Figure 3.** Hybrid larch (Larix x eurolepis) variety REVE VERT somatic embryogenesis: performance at each step.*

Subsequently plants, during their vigorous growth phase, are directly transferred from the Petri dishes to a potting mix under shade house conditions (Lelu et al. 1994b). This simple procedure allowed high plantlet survival (79% after 8 months in the shade house) before their transfer to the nursery (Lelu-Walter and Pâques 2009).

Somatic embryogenesis has been successfully used to produce the new hybrid larch *Larix* x *eurolepis*, variety REVE-VERT (Figure3) registered in 2005 and produced by a cross of one European larch clone with a polymix of 12 Japanese larch clones (Lelu-Walter and Pâques 2009). Microsatellite markers are useful for searching paternity (checking the heredity), studying genetic diversity (Nardin et al. 2015) and structure of populations (Wagner 2013). For larch two multiplexes with 7 and 6 microsatellite markers have been developed (Wagner et al. 2012). These markers show a gradient of polymorphism from 4 to 15 alleles in our populations. These 13 markers are available to discriminate close individuals, as full-sib lines. For each embryogenic line obtained, the father has been identified without doubt using the 13 markers (Table 3).

5.2 Environmental conditions and physiological status of somatic embryos: are they similar to those of their zygotic counterpart?

Water availability is a key factor for pine somatic embryo maturation (see chapters in this book dealing with pine SE) and appeared to control the development of Japanese and hybrid larch somatic embryos. For hybrid larches, use of a high gellan gum concentration (8 g L^{-1}) in the maturation medium instead of a low one (4 g L^{-1})resulted in an improved physiological status of the somatic embryos considering their dry weight and water content (Teyssier et al. 2011). The accumulation of proteins has also been impacted. After 6 weeks of maturation somatic embryos that developed on 8 g L^{-1} medium had a higher protein content than their counterparts on the 4 g L^{-1}, a difference that was not significant after 8 weeks (Table 4). The identified differentially abundant proteins showed a reduction in abundance of enzymes involved in the glycolysis pathway and HSPs. Interestingly comparing proteomes, the identified proteins suggested that the embryos were more stressed when they were matured on 4 than on 8 g L^{-1} of gellan gum (Teyssier et al. 2011). These results strengthened the choice to mature hybrid larch somatic embryo with 8 g L^{-1} gellan gum.

In conifers the effect of abiotic factors, such as light during somatic embryo maturation, are not commonly studied. Although zygotic embryos develop in the dark, researchers generally specify light conditions for somatic embryogenesis but on what basis? In larch routine maturation protocols are realized either in light (Kim and Moon 2007) or in darkness (Lelu-Walter and Pâques 2009). Recently the effect of environmental conditions (light vs darkness)

has been investigated during somatic embryo maturation of hybrid larch. Morphogenesis of somatic embryos was not different in light or dark: they had a

Table 3.Paternity assignment of embryogenic line N23 of hybrid larch (Larix x *eurolepis) of the REVE VERT variety (Lelu-Walter and Pâques 2009): European larch clone (mother 1) crossed with a polymix of 12 Japanese larch clones (putative father A-L).*

	Identity	bcLK_189		bcLK_211		bcLK_228		bcLK_253		bcLk_263		Ld_101		Ld_30		Ld_31		Ld_42		Ld_45		Ld_50		Ld_56		Ld_58	
	LOD score*	0.475		0.641		0.644		0.618		0.724		0.533		0.196		0.509		0.270		0.072		0.170		0.152		0.631	
	PIC**	0.793		0.881		0.883		0.872		0.915		0.832		0.554		0.818		0.638		0.348		0.509		0.493		0.878	
Mother	1	141	155	null	187	186	196	206	218	214	226	182	187	117	119	136	136	180	182	206	206	null	176	236	238	null	161
Progeny	N23***	154	155	187	205	null	196	214	218	214	216	187	198	117	117	null	136	173	182	206	212	null	169	231	236	141	161
Putative father	A	141	172	187	201	190	192	202	212	192	212	187	204	115	115	114	130	167	175	212	212	157	169	223	223	157	157
Putative father	B	141	172	207	211	null	186	220	222	190	210	187	190	115	117	null	134	167	167	212	212	169	169	231	231	141	143
Putative father	C	141	162	191	199	188	192	218	222	200	200	184	184	115	115	null	null	173	175	210	212	157	169	231	231	131	147
Putative father	D	141	172	187	191	190	194	198	202	200	202	184	184	117	117	114	138	175	175	209	212	169	173	231	231	149	149
Putative father	E	154	172	189	201	null	194	218	220	188	200	184	192	115	115	null	132	173	173	212	212	157	157	231	231	141	143
Putative father	F	141	154	191	215	191	200	202	212	202	222	190	204	115	115	132	136	167	173	212	212	169	169	231	231	null	null
Putative father	G	141	170	197	211	196	198	204	206	208	234	184	204	115	115	114	138	173	175	212	212	169	169	231	232	null	null
Putative father	**H**	**154**	**174**	**187**	**205**	**null**	**194**	**212**	**214**	**208**	**216**	**198**	**198**	**117**	**117**	**null**	**114**	**173**	**173**	**212**	**212**	**169**	**169**	**231**	**231**	**141**	**143**
Putative father	I	141	162	187	191	190	196	206	222	192	212	198	204	null	null	114	114	173	173	212	214	157	169	231	231	143	143
Putative father	J	141	141	195	215	193	196	202	210	206	208	187	192	115	115	131	136	173	173	212	212	157	169	231	231	137	145
Putative father	K	152	174	203	203	192	192	202	202	196	212	190	204	115	115	138	138	167	173	212	212	169	169	231	231	141	145
Putative father	L	148	158	205	207	184	184	218	222	196	218	189	200	119	119	134	136	167	175	212	212	157	169	null	null	139	145

*LOD: Lod Score; **PIC: Mean polymorphic information content; *** putative father: H.

***Table 4**: Quantitative analysis of total proteins in somatic embryos of hybrid larch during maturation according to gellan gum concentration (4 vs 8 g L^{-1}).*

Maturation time (weeks)	Protein quantity*			
	µg mg^{-1} FW		µg $unit^{-1}$	
	4 g L^{-1}	8 g L^{-1}	4 g L^{-1}	8 g L^{-1}
1	16,68 ± 2.7 a	19,45 ± 5.6 a	n.d.	n.d.
3	22,05 ± 6.3 a	39,53 ± 7.8 ab	7,37 ± 2.1 α	7,81 ± 1.5 α
6	66,74 ± 11.6 bc	108,54 ± 9.9 d	68,44 ± 7.9 β	80,45 ± 7.3 βγ
8	83,25 ± 28.9 cd	96,44 ± 23.7 cd	106,26 ± 37 γ	85,58 ± 21.0 βγ

* Values are means ± standard error of six repetitions. In each column, significant differences (P <0.05) in a multiple comparison of means are indicated by different letters. . FW: fresh weight; n.d.: not determined.

full set of organs, i.e., cotyledons, hypocotyl, and embryonal root cap (von Aderkas et al. 2015). However light had a negative effect on protein accumulation but a positive effect on phenol accumulation (quercetrin production, von Aderkas et al. 2015). In hybrid larch, maturation in darkness promoted a development of somatic embryos that was similar to that of zygotic embryos whereas light conditions affected protein and phenolic compound accumulation, especially in the embryonal root cap. Considering the accumulation pattern of storage reserves such as proteins, zygotic embryos and mature somatic embryos showed similarities in (i) their protein profile, (ii) the presence of storage proteins vicilin -like- and legumin -like protein, (iii) their total protein content levels (Teyssier et al. 2014). Thus an improved maturation protocol leads formation of mature somatic embryos that most closely resemble zygotic embryos in their morphology, anatomy, and protein contents. However, the difference between somatic and zygotic embryos in their plant growth regulator content (auxin, cytokinin, ABA) stressed that mature somatic embryos are produced in a fundamentally different physiological context than zygotic embryos (von Aderkas et al. 2001). Indeed somatic embryos lack a storage tissue and require non-physiological levels of ABA to mature properly.

5.3 When to harvest cotyledonary somatic embryos for germination?

Current maturation protocols produce “mature” somatic embryos that morphologically resemble zygotic embryos and are kept in the maturation phase during an arbitrary length of time before subsequent germination. Such an empirical approach does not give any information concerning the quality of somatic embryos needed to achieve maximal plant conversion rates because the potential vigor of the emblings is conditioned by the quality of the embryos (Terskikh et al. 2005; Businge et al. 2013). Therefore, we need to develop markers to assess the quality of somatic embryos. During maturation concomitantly to a dry weight increase, the amount of protein increased reaching a maximum at 8 weeks followed then by a decrease (Teyssier et al. 2014). At this developmental stage, called « late cotyledonary embryo stage», changes in the miRNAs expression appeared very important (Zhang et al. 2012). These proceed to inactivate transcripts involved in various maturation processes such as lignification and thickening of the cell wall, or in energy metabolism during embryogenesis. The activity of certain enzymes can also be used to track changes in metabolism during late embryogenesis and then to identify the molecular status of the embryos (Bailly et al. 2001). In hybrid larch while the activity of enzymes involved in cellular metabolism does not change between 6 and 9 weeks of maturation, the enzymes involved in the anti-oxidative protection of cells have a peak between 6 and 8 weeks of cultures (Table 5); in Japanese and Chinese larches, activities of these enzymes increased as embryos get older (Zhang et al. 2010; Zhao et al. 2015). A

recent proteomic study of the development of somatic embryos gave also novel insights into this process in larch and provided identification of new markers (Zhao et al. 2015).

Table 5*: Change in enzyme activities in somatic embryos of hybrid larch during maturation on 8 g L^{-1} gellan gum. Results are expressed as the percentage of the activity measured at 6 weeks of maturation. Values are means of 3 measurements ± standard error.*

Enzyme activity	Functional group*	Week of maturation			
		6	7	8	9
enolase	cellular metab.	100 ± 31.0	57.55 ± 31.2	51,90 ± 9.0	79,94 ± 22.1
glyceraldehyde 3 phosphate dehydrogenase	cellular metab.	100 ± 44.3	127,79 ± 24.0	62,57 ± 5.0	66,82 ± 23.8
invertase	cellular metab.	100 ± 40.0	71,60 ± 21.3	204,53 ± 25.7	49,56 ± 19.6
ascorbate peroxydase	antiox. protect.	100 ± 20.3	100,58 ± 6.10	90,95 ± 10.0	101,07 ± 18.40
catalase	antiox. protect.	100 ± 13.2	112,40 ± 14.70	113,25 ± 9.80	83,20 ± 26.4
glutathion peroxydase	antiox. protect.	100 ± 3.4	116,21 ± 15.0	80,50 ± 32.3	113,55 ± 22.10
glutathion reductase	antiox. protect.	100 ± 38.8	190,97 ± 18.4	115,69 ± 28.0	183,05 ± 71.0
pyruvate kinase	antiox. protect.	100 ± 31.1	89,65 ± 27.4	75,36 ± 28.8	133,66 ± 23.8
superoxyde dismutase	antiox. protect.	100 ± 4.2	86,29 ± 11.2	77,14 ± 12.2	83,00 ± 15.6

* Cellular metab: cellular metabolism; antiox. protect.: antioxidative protection.

Maturation duration appeared to influence the subsequent step, i.e., somatic embryo germination. In hybrid larch, extension of the maturation period in the presence of ABA resulted in a significant decrease in both germination and plantlet frequencies (Label and Lelu 1994) that has been correlated with an increase of the *in planta* ABA content (Lelu and Label 1994; Label and Lelu 2000). Consequently a desiccation treatment (1 week at 4°C under a high relative humidity, 98% RH) was applied to cotyledonary somatic embryos matured in presence of ABA. After drying, germination had become synchronised (at a frequency between 89 and 100%) and plantlet recovery had improved (87%, Lelu et al. 1995). Desiccation treatment resulted in a decrease in endogenous ABA content of the somatic embryos (Dronne et al. 1997) while the final water content approximated that of stored seed (Lelu et al. 1995). Desiccation not only enhanced germination capacity but it may also be considered as a method of storage. More recently in Japanese larch gene regulation has been investigated during embryo dormancy and germination showing different expression patterns of miRNAs

(Zhang et al. 2013).

Therefore, it appears that when somatic embryos become cotyledonary, they no longer change morphologically, even though physiological and molecular changes are taking place with accumulation of protein energy reserves and an increase of dry weight and a reduction of the water content (Teyssier et al. 2011). This implies a modification of enzyme activities and of their regulation. For hybrid larch 8 week old somatic embryos seem to be at their maximum quality. Beyond the 8 weeks period phenomena of storage protein hydrolysis and oxidation quickly appear.

6. **Somatic embryogenesis as a tool for breeding programs**

All the progress obtained in hybrid larch SE, have contributed to the development of an improved procedure leading to the routine production of emblings. Among conifer species, somatic embryogenesis of *Larix* is becoming a model for its multiple uses and its integration into breeding programs is now undertaken for the clonal propagation of material improved by breeding of hybrid larch *Larix* x *eurolepis*, variety REVE-VERT (Lelu-Walter and Pâques 2009, Figure 3). We believe that somatic embryogenesis should influence breeding strategies by offering an alternative tool for accelerated mass-production of plants from improved genotypes (full-sib hybrid family). Another use could be to rely on somatic embryogenesis as an alternative to rooted cuttings to increase precision in progeny testing: vegetative propagation of hybrid full-sib families would allow testing them over a significantly greater number of sites for a better genetic evaluation and integration of GxE. Testing the same genotypes in contrasting environments will allow evaluating their phenotypic plasticity. Indeed, due to the low actual reproductive success in larch, the number of sibs per full-sib-family is usually much reduced, especially when one has to use factorial/diallel mating designs.

Another use of somatic embryogenesis in the context of breeding is exemplified by one objective of the Trees4Future Research infrastructure network (http://www.trees4future.eu/), namely to provide genetically stable genotype references to support breeders and genetic research and activities at various levels (e.g. genetic control to estimate genetic gains; provide contrasted genotypes for benchmarking subjective scoring-scales; help to establish pan-European plots to monitor impact of abiotic and biotic factors on tree characteristics). These genotypes can be delivered at the appropriate time through vegetative propagation by somatic embryogenesis thanks to cryopreservation.

Besides its high potential for mass-propagation, the main revolution of somatic embryogenesis in the context of vegetative propagation, is linked to the flexibility offered by the possibility to cryopreserve embryogenic lines and

maintain them juvenile (Figure 2). Concretely, this means that whereas lines (clones) are evaluated in the field for further selection some years or decades later, juvenile stock can be re-activated at any time for mass-propagation.

The strength of somatic embryogenesis is probably best optimized when a few elite-lines are mass-produced at a time. If clonal forestry has found favourable echoes in some countries, within a European forestry context more genetically diverse plantations are favoured, meaning the deployment of several tens of clones at a time. For hybrid larch in France (as for Sitka spruce in UK), full-sib-family forestry is considered to offer an acceptable genetic diversity level. Therefore, to alleviate the difficulty of somatic embryogenesis to handle many lines simultaneously, a combination of somatic embryogenesis and of bulk propagation by cuttings is a possible option. Juvenile stock plants regularly produced from cryopreserved elite-lines by somatic embryogenesis will be mass-propagated by cuttings.

Because genetic gains are often linked to the age at which selection of superior genotypes is possible, the perspective to vegetatively mass-propagate elite adult trees remains for breeders a dream and for biotechnologists a challenge (see chapter by Klimaszewska et al. in this book).

7. Conclusions and perspectives

Somatic embryo maturation is a complex process triggered by many factors. By combining the qualitative and quantitative results obtained with Japanese and hybrid larches, a maturation medium with ABA (60µM) plus 0.2M either sucrose or maltose and 8 g L^{-1} gellan gum is now routinely used to promote somatic embryo maturation. We found that the protein content is a reliable indicator of the physiological maturity of larch somatic embryos. When necessary, desiccation could be applied to somatic embryos in order to synchronize germination and to improve the germination and plantlet formation frequencies. For hybrid larch, the somatic embryogenesis process has been enough refined to be used on a large scale. The rapidity with which new material can be produced and the high potential for amplification make somatic embryogenesis a powerful and flexible tool for release of improved varieties. Full success nevertheless conditioned depends on several requirements posed by breeders. As with any other propagation system, breeders are firstly concerned by the integrity of the propagated, improved variety both in terms of its mean performance and of its genetic diversity. Firstly, as has been demonstrated for rooted cuttings, trees produced by somatic embryogenesis must show no detrimental abnormalities and must behave like or even better than seedlings in terms of growth, architecture, stability and maturation. Confirmation of growth behaviour comparable to that of seedlings is still needed. Tests are now in progress to compare the agronomic

behaviour of emblings with other material (seedlings, cuttings). Secondly, the genetic diversity of the material thus far released (Forest Reproductive Material) has to be enlarged and should not be limited to the few lines available today. A minimum number (10) of successful embryogenic lines that are genetically diverse, should be propagated to compose a multiclonal variety. Finally, practical questions related to logistic aspects (e.g., how many families and individuals per family can be practically managed during the different steps of the technique?) and to the cost of propagation should be properly addressed. As already mentioned above, somatic embryogenesis from adult trees remains a challenge which forces us to try to achieve a better understanding of the molecular biology of embryo development (Vestman et al. 2011, Morel et al. 2014; Yakovlev et al. 2014).

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9. Authors contribution.

MALW conceived in the design of the study and its coordination, and drafted the manuscript. CT carried out the protein analysis and drafted the manuscript. LP conceived the design of the study and drafted the manuscript. VG carried out the paternity assignment analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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