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Research article

Simplified and improved somatic embryogenesis of hybrid larches (*Larix* x *eurolepis* and *Larix* × *marschlinsii*). Perspectives for breeding

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Keywords:
Breeding programme / clonal propagation / *Larix* × *eurolepis* / *Larix* × *marschlinsii* / somatic embryogenesis

Abstract

• Development of clonal propagation method, such as somatic embryogenesis, has numerous applications such as mass-production of genetically improved plants and the amenability of embryogenic cultures to cryogenic storage. Since the 90's, researchers at INRA have engaged in research on somatic embryogenesis in *Larix* species (*Larix* × *eurolepis, Larix* × *marschlinsii*).

• The aim of this work was to improve and to simplify all steps of somatic embryogenesis and to apply this protocol to the new hybrid variety REVE-VERT.

• The somatic embryogenesis initiation frequency from immature zygotic embryos was high (65%) on a medium with reduced plant growth regulator concentrations (2.2 μM of 2.4-dichlorophenoxyacetic acid and 2.3 μM of 6-benzyladenine). Simplified cryopreservation method (no need of programmable freezer) of the embryonal masses resulted in 100% recovery of cryopreserved lines. Maturation of a large number of somatic embryos was greatly improved when embryonal masses were dispersed on filter paper placed on medium containing high concentration of gellan gum (8 $g \cdot L^{-1}$). Under these conditions, 94% of the lines matured somatic embryos that developed into plantlets. Clearly ageing and cryopreservation did not reduce embryogenic potential of embryonal masses.

• Requirements for the effective integration of somatic embryogenesis into the larch breeding programme are discussed.

Mots-clés :
programme d'amélioration / multiplication clonale / *Larix* × *eurolepis* / *Larix* × *marschlinsii* / embryogenèse somatique

Résumé – Simplification et amélioration de l'embryogenèse somatique des mélèzes hybrides (*Larix* × *eurolepis* et *Larix* × *marschlinsii*). Perspectives pour l'amélioration.

• Le développement de méthode de multiplication clonale, telle l'embryogenèse somatique, a de nombreuses applications comme la production en masse de plants génétiquement améliorés et la disponibilité des cultures embryogènes en cryoconservation. Depuis les années 1990, l'INRA a engagé des recherches en embryogenèse somatique des mélèzes (*Larix* × *eurolepis, Larix* × *marschlinsii*).

• L'objectif du travail a été de simplifier et d'améliorer chaque étape de l'embryogenèse somatique, et d'appliquer ce protocole à la nouvelle variété de mélèze hybride REVE-VERT.

• L'initiation de l'embryogenèse somatique à partir d'embryons zygotiques immatures est obtenue à des fréquences élevées (65 %) avec un milieu de culture contenant des concentrations hormonales réduites (2,2 μM d'acide 2.4-dichlorophenoxyacetic et 2,3 μM de 6-benzyladenine). Une méthode simplifiée de congélation (sans l'utilisation d'un congélateur programmable) des masses embryogènes permet la survie de 100 % des lignées congelées. La maturation d'un grand nombre d'embryons somatiques a été fortement améliorée lorsque les masses embryogènes ont été étalées sur filtre papier placé sur milieu de culture à forte concentration en gelrite (8 $g \cdot L^{-1}$). Sous ces conditions, 94 % des lignées embryogènes régénèrent des embryons somatiques matures qui se développent en plantes. Clairement l'âge et la cryoconservation des lignées embryogènes ne réduisent pas leur potentiel embryogène.

• Les besoins pour une intégration efficace de l'embryogenèse somatique au programme d'amélioration du mélèze, sont discutés.

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1. INTRODUCTION

The genus *Larix* is important in reforestation programmes due to its fast growth, wide ecological plasticity and desirable wood properties (Gower and Richards, 1990). In Europe, the two main species commonly used in breeding programme are European larch (*L. decidua*) and Japanese larch (*L. kaempferi)*. Interspecific hybridization is used to combine favourable traits from both parent species and it represents a major strategy in larch breeding. Compared to the parental species, the hybrids such as *L.* × *eurolepis* (*L. decidua* × *L. kaempferi*) and *L.* × *marschlinsii* (*L. kaempferi* × *L. decidua*) are known for their remarkable vigour as well as for their superior stem form and site adaptation (Pâques, 1989; 1992).

In France, the larch breeding programme started nearly 50 years ago at INRA-Orléans but the first hybrids produced by controlled crosses were obtained only recently in 1979. The first hybrid variety (REVE-VERT) was registered in 2005. Meanwhile, intensive research on traditional mass-propagation techniques was conducted for hybrid seed production (supplemental pollination, indoor orchard, F_2-hybridisation orchard) and vegetative propagation by rooting of cutting.

New genetic combinations of favourable characters can be achieved by conventional breeding techniques but poor seed set in production orchards often limits the deployment of hybrid (Owens, 1995). Advances in plant biotechnology offer new opportunities to overcome these limitations especially through the application of somatic embryogenesis (SE) (Klimaszewska et al., 2007). Clonal propagation using SE has numerous applications and has advantages over conventional rooted cuttings. Plant regeneration by SE constitutes a tool for research such as for study of gene function. SE can also contribute to increase selection efficiency and facilitate mass deployment of improved varieties. SE has, in many instances become the method of choice for clonal propagation of conifers (Sutton, 2002), due to its high and sustained productivity and the amenability of embryogenic cultures to cryogenic storage (Park, 2002).

In hybrid larch, SE has been first reported in 1989 for *L.* × *eurolepis* Henry by Klimaszewska (1989) and subsequently for its reciprocal *L.* × *marschlinsii* Coaz by Lelu et al. (1994a; 1994b). Integration of SE into the larch breeding programme is now undertaken for the clonal propagation of the new variety REVE-VERT and newly selected ones. Indeed, SE influences hybrid larch breeding strategies by offering an alternative tool for accelerated production of superior hybrids identified through clonal testing. To achieve this objective, we need both to improve the number and quality of somatic embryos and simplify the process. Presently, there are two major limitations to the large-scale propagation of hybrid larch through SE: (1) the low initiation rates and (2) the poor somatic embryo maturation efficiency for a number of embryogenic lines. In addition, we have to answer the following new question arising from the technique itself: cryopreservation offers real new perspectives for long-term conservation and reactivation of lines at any time (Park et al., 1998) but we have to study its impact on the genetic stability (i.e. ability to regenerate somatic embryos).

This paper presents a significantly improved procedure for SE of hybrid larches leading to the production of somatic embryos in high numbers. The new protocol was applied for the propagation of the new hybrid variety REVE-VERT. Integration of SE into the breeding programme and future research needs are also addressed.

2. MATERIAL AND METHODS

2.1. Initiation of somatic embryogenesis from variety REVE-VERT (experiment 1)

REVE-VERT variety produced by one European larch clone crossed with of polymix of 12 Japanese larch clones, was obtained in 2004, 2005 and 2006 at INRA Orléans, France. The immature cones were collected June on 7th, 10th and 13th in 2004, 2005 and 2006, respectively, and immature seeds were excised according to the method described by Lelu et al. (1994a).

Briefly, cones were surface-sterilized for 10 min with 95% ethanol containing a drop of Tween 20 and then dried in the laminar flow unit. Scales with immature seeds were removed, from the base toward the cone apex; seeds were picked with sterile forceps and placed on a sterile surface. During this procedure, the immature seeds were not disinfected, yet the contamination rate of the cultures was less than 0.1%. Seeds were extracted when the zygotic embryos were at an early stage of late embryogeny (von Aderkas et al., 1991). At this developmental stage, megagametophytes that contained zygotic embryos were carefully removed from the seed coat, nucellus and megaspore wall, and placed on initiation medium. From previous results (Lelu et al., 1994a), plant growth regulator (PGR) concentrations in the initiation medium were reconsidered. Immature explants were cultured on MSG medium (Becwar et al., 1990) containing 1.45 g·L^{-1} L-glutamine (SIGMA) supplemented with 2.4-dichlorophenoxyacetic acid (2,4-D) at either 9 or 2.2 µM, and 6-benzyladenine (BA) at either 4.4 or 2.3 µM. The medium with the higher concentrations of 2,4-D and BA, was designated as MSG-S (for standard concentration of PGRs), and that with lower concentrations of the two compounds as MSG-L (for low PGRs). Subsequently another basal medium was tested consisting in modified Litvay's medium (mLV, Litvay et al., 1985) by reducing the concentration of macro elements by 50% (except iron and EDTA) and adding 1 g·L^{-1} casein hydrolysate (enzymatic, SIGMA, CH) and 0.5 g·L^{-1} L-glutamine. This medium was supplemented with 2.2 µM of 2,4-D and 2.3 µM of BA and designed as mLV-L. The media containing 60 mM sucrose were solidified with 4 g·L^{-1} gellan gum (Phytagel™, SIGMA). The pH of the media was adjusted to 5.8 ± 0.1 prior to autoclaving. All experiments were conducted in 90 × 20 mm Petri dishes. The explants, usually 10 per Petri dish, were cultured for up to 10 weeks in darkness at approximately 25 °C and were not subcultured during the whole period. They were considered as having initiated somatic embryogenesis if embryonal masses (EM) could be observed by naked eye. After 10 weeks, EM that showed continuous growth and produced amounts of fresh mass (f.m.) sufficient for subculture was considered to have "proliferated".

2.2. First and subsequent subcultures of embryogenic tissue

The age of the embryogenic tissue was set to 0 the day of the first subculture. The EM were sub-cultured in clumps every 2 weeks onto fresh proliferation medium which consisted of basal MSG medium supplemented with 2,4-D (9 μM), BA (2.3 μM), 60 mM sucrose and solidified with 4 g·L^{-1} gellan gum. Each Petri dish, containing 15 EM, was incubated in darkness at approximately 25 °C. In order to rapidly multiply the embryogenic tissue, we applied the proliferation method previously developed for pine species (Lelu-Walter et al., 2006; 2008). Proliferating EM after a week, were collected and suspended in 4 to 5 ml of liquid proliferation medium, vigorously shaken to break up the tissue pieces into a fine suspension, and poured in a thin layer onto a filter paper (Whatman N° 2, diameter 7 cm) in a Büchner funnel. Low-pressure pulse was applied to drain the liquid, and the filter paper with attached cells was placed on the surface of fresh proliferation medium. The EM density per filter was approximately 300 mg f.m.

2.3. Cryopreservation

The simplified cryopreservation protocol previously published for other conifer species (Lelu-Walter et al., 2006; 2008) has been adapted. Briefly, 3 g f.m. EM cultured on filter paper for 1 week were suspended in 12 mL of liquid proliferation medium supplemented with 0.4 M sucrose for 24 h. Subsequently, 3 mL of dimethylsulphoxide (DMSO, SIGMA) was added to the suspension on ice (final DMSO concentration 10%). After 1.5 h, 1 mL of suspension was transferred into cryovials (Nalgene™ Cryo 1 °C Freezing Container) that was placed in a freezer at –80 °C for 2 h. The vials were then submerged and stored in liquid nitrogen. Of 12 vials frozen per embryogenic line, two were thawed after 24 h to test the survival of cells.

2.4. Maturation of somatic embryos

Experiments were conducted with one line (69-18) of hybrid larch *Larix* × *marschlinsii* obtained in 1992 through secondary somatic embryogenesis (Lelu et al., 1994c) and with 6 lines of hybrid larch *Larix* × *eurolepis* from REVE-VERT family initiated in 2003 (N23), in 2004 (P1, P21, P22) and 2005 (Q21, Q42). The experiments were designed to investigate the effects of various factors including the cultural method, the light, the gellan gum concentration, on the production of mature somatic embryos.

The maturation procedure comprised two successive steps (Lelu et al., 1994a). One week after subculture on the proliferation medium, pieces of EM were first transferred to PGR-free MSG medium containing activated charcoal (AC, Merck, at 10 g·L^{-1}), 0.1 M sucrose and solidified with 4 g·L^{-1} gellan gum. Petri dishes were placed for one week under cool-white light (Philips) at a photon fluence density of 10 μmol m^{-2} s^{-1} at 24/21 ± 1 °C under a 16-h photoperiod. Thereafter, EM were transferred to the second maturation medium containing 0.2 M sucrose, 1 μM indolebutyric acid (IBA) and 60 μM *cis-trans* (±)-abscisic acid (ABA). At this time, the light intensity was increased to 20 μmol m^{-2} s^{-1}. After 5 weeks of culture, somatic embryos were counted and the embryogenic potential was estimated (number of somatic embryos per g f.m.).

IBA, ABA and L-glutamine were filter-sterilised and added to cooled autoclaved media. Exceptions to the above and features specific to each experiment are listed below.

2.5. Improvement and simplification of the maturation of somatic embryos (experiment 2)

A first experiment was designed to investigate the effects of the cultural method of EM on somatic embryo production. Embryonal masses of line 69-18 were cultured in clumps (50 to 90 mg f.m. each), 6 per Petri dish or as a thin layer dispersed onto a filter paper as for pine species (Lelu-Walter et al., 2006; 2008). Seven days after subculture on the proliferation medium, pieces of actively growing EM were weighed, dispersed into liquid MSG medium without PGR, and distributed onto a filter paper disc as previously described for the proliferation step. Filter paper discs with dissociated EM (approximately 200 mg f.m.) were placed for 1 week on PGR-free MSG medium supplemented with activated charcoal and then transferred to ABA containing medium as previously described. Experiment was repeated twice and the number of Petri dishes per cultural method varied from 6 to 18 for a total of 45 Petri dishes. Based on the results of this experiment, maturation was subsequently performed with EM dispersed on a filter paper disc.

The effect of the light on somatic embryo maturation was also considered. The Petri dishes were either placed in the dark during the entire maturation procedure or in the light as previously described. Experiment was repeated twice with 5 to 10 Petri dishes per condition for a total of 30 Petri dishes.

Recent results obtained in pine species showed that the increase of the gellan gum concentration resulted in an improved maturation (Lelu-Walter et al., 2006; 2008). In consequence, this factor was tested with 5 REVE-VERT lines (N23, P21, P22, Q21, Q42). Gellan gum concentration of ABA maturation medium, varied 4, 8, and 10 g·L^{-1} and the somatic embryo production was assessed. The number of Petri dishes varied from 8 to 10 for a total of 139.

2.6. Effect of age and cryopreservation on embryogenic potential (experiment 3)

The effect of the age of the embryogenic tissue on the embryogenic potential was assessed for REVE-VERT lines N23, P1 and P22 at age varying from 4 to 21 months. The number of Petri dishes per age varied from 5 to 12 for a total of 38 Petri dishes. This age effect could be tested with the line 69-18 routinely used in our laboratory, and that has been sub-cultured for a long period. Indeed, maturation experiments were realised in 1997, 1999, 2000 and 2001, i.e., EM aged respectively 5, 7, 8 and 9 years in culture. The number of Petri dishes per age varied from 6 to 10 for a total of 27 Petri dishes.

Finally, as lines were cryopreserved and routinely used for the experiments, maturation experiment was realised to assess the effect of cryopreservation on somatic embryo production. The line 69-18 cryopreserved in 1999 was thawed in 2002 (69-18c) i.e., after 3 years of cryopreservation. Maturation experiment was repeated twice, with 5 to 8 Petri dishes per repetition for a total of 19 Petri dishes.

2.7. Maturation and plant production of hybrid larch *Larix* × *eurolepis* REVE-VERT variety (experiment 4)

Based on the results of previous experiments, an improved maturation protocol was applied to promote somatic embryo development in 24 lines of REVE-VERT variety. EM dispersed on filter paper were transferred to PGR-free MSG medium containing activated charcoal before being transferred onto ABA MSG medium solidified

with 8 g·L^{-1} of gellan gum for 5 weeks without subculture. Maturation was conducted in darkness and repeated 2 to 4 times, with number of Petri dishes varied from 3 to 10 per line and repetition. The number of cotyledonary somatic embryos produced after 5-week period was counted in each of the 282 Petri dishes.

Subsequent germination and plant regeneration were performed as previously described (Lelu et al., 1994b). Briefly, cotyledonary somatic embryos were removed from the EM after 5 to 6 weeks of culture in the presence of ABA and cultured on PGR-free MSG medium containing 90 mM sucrose and 4 g·L^{-1} gellan gum. Somatic embryos were considered germinated as soon as their radicle emerged. The Petri dishes were placed for 1 week in darkness to promote hypocotyl elongation. Somatic embryos were then exposed to a 16-h photoperiod (10 µmol m^{-2} s^{-1}) at 24/21 °C day/night temperatures. Germinated somatic embryos were considered as plantlets as soon as their epicotyl developed. Effect of gellan gum concentration of the maturation medium on subsequent somatic embryo development was assessed for 3 lines (N23, P21, P22). Germination and plantlet frequencies were recorded for a total of 43 Petri dishes.

After 6 to 7 weeks, the plantlets were directly acclimatized according to the method previously described (Lelu et al., 1994b) at INRA, Orléans by mid May. The survival was assessed 3 months after transfer to the shade house.

2.8. Statistical Analysis

The data mostly consisted of the number of somatic embryos produced under given treatments: they were first expressed as number of somatic embryos per g of fresh mass. In some instances, they were expressed in percentage of success as in experiments 1 and 4. In both cases, data normality was checked and the significance of the different factors such as treatments, lines, was tested by one-way or two-way analysis of variance on individual data. When more than two levels were present for a significant factor, Scheffé test was used for comparison of means.

3. RESULTS

3.1. Initiation of somatic embryogenesis from variety REVE-VERT (experiment 1)

The first evidence of SE initiation was visible on some explants after 3 weeks of culture with the majority of explants reacting after 5–6 weeks. Most of the initiated embryonal masses proliferated after 6 to 10 weeks. Results obtained in 2004 on a medium (MSG) did not show any significant differences between the standard (MSG-S) and the reduced (MSG-L) plant growth regulator concentrations treatments on both initiation rate and proliferation rates. Respective values for initiation were 32 ± 9% (MSG-S) and 35 ± 22% (MSG-L) and for proliferation, 47 ± 26% (MSG-S) and 63 ± 34% (MSG-L). Based on these results, the lowest plant growth regulator concentration was used the following years and two basal media (MSG-L and mLV-L) were compared. Results were consistent over years (no year effect): mLV-L medium gave higher initiation and proliferation rates than MSG (Tab. I) but differences were significant only for initiation rates ($p < 0.00001$).

Table I. Mean initiation and proliferation rates (± one standard-deviation) of EM from immature zygotic embryos of *Larix* × *eurolepis* over years on two media (MSG, mLV) with low plant growth regulator concentrations (L).

Medium	Year		
	2004	2005	2006
Initiation			
MSG-L	34.6 ± 21.8	28.0 ± 14.3	37.7 ± 17.3
mLV-L	53.6 ± 5.1	60.1 ± 14.8	64.7 ± 17.1
Proliferation			
MSG-L	63.1 ± 33.8	58.4 ± 34.8	74.6 ± 23.4
mLV-L	67.5 ± 10.6	67.3 ± 19.6	78.0 ± 15.9

3.2. Proliferation of EM

A new multiplication method based on EM spread over a filter paper disc, was developed. Under these conditions, EM grew nicely and yielded high quantities of reactive material. From 0.3 g fresh weight spread at day 0, the amount of tissue could increase to over 3 g after 1 week of culture. This proliferation method was thus routinely used to produce actively growing and uniform material for cryopreservation and somatic embryo maturation.

3.3. Cryopreservation

Two weeks after thawing, all frozen lines survived and started to proliferate. After 4 weeks, enough material was produced to perform proliferation. The simplified cryopreservation technique (no need for programmable freezer) resulted in 100% recovery of all cryopreserved lines. This consisted of 60 lines with 31 from REVE-VERT. Survival and recovery of EM were improved when DMSO was added to the suspension (10% final concentration) and when sucrose 0.4 M was used instead of sorbitol 0.4 M (data not shown).

3.4. Improvement and simplification of the maturation of somatic embryos (experiment 2)

Different maturation procedures were tested in order to get high number of cotyledonary somatic embryos. We first applied the method previously developed for hybrid larch involving the transfer of individual EM clumps onto maturation medium (Lelu et al., 1994a). Then, we matured EM dispersed onto a filter paper as a thin layer (as for proliferation). This filter treatment drastically increased the number of somatic embryos per g of f.m. to an average of 1410 (± 746) embryos from an average of 573 (± 196) for the clump method. The differences were highly significant ($p < 0.0001$).

In darkness, somatic embryos remained yellowish, compared to those matured in light that produced anthocyanins. Maturation of the embryos in the dark proved to be as efficient as with light. The numbers of somatic embryos obtained per g of f.m. did not differ significantly between dark and light (903 ± 345 and 1006 ± 493 respectively).

Increasing the gellan gum concentration improved the morphological aspect of the somatic embryos: on 8 and 10 g·L^{-1},

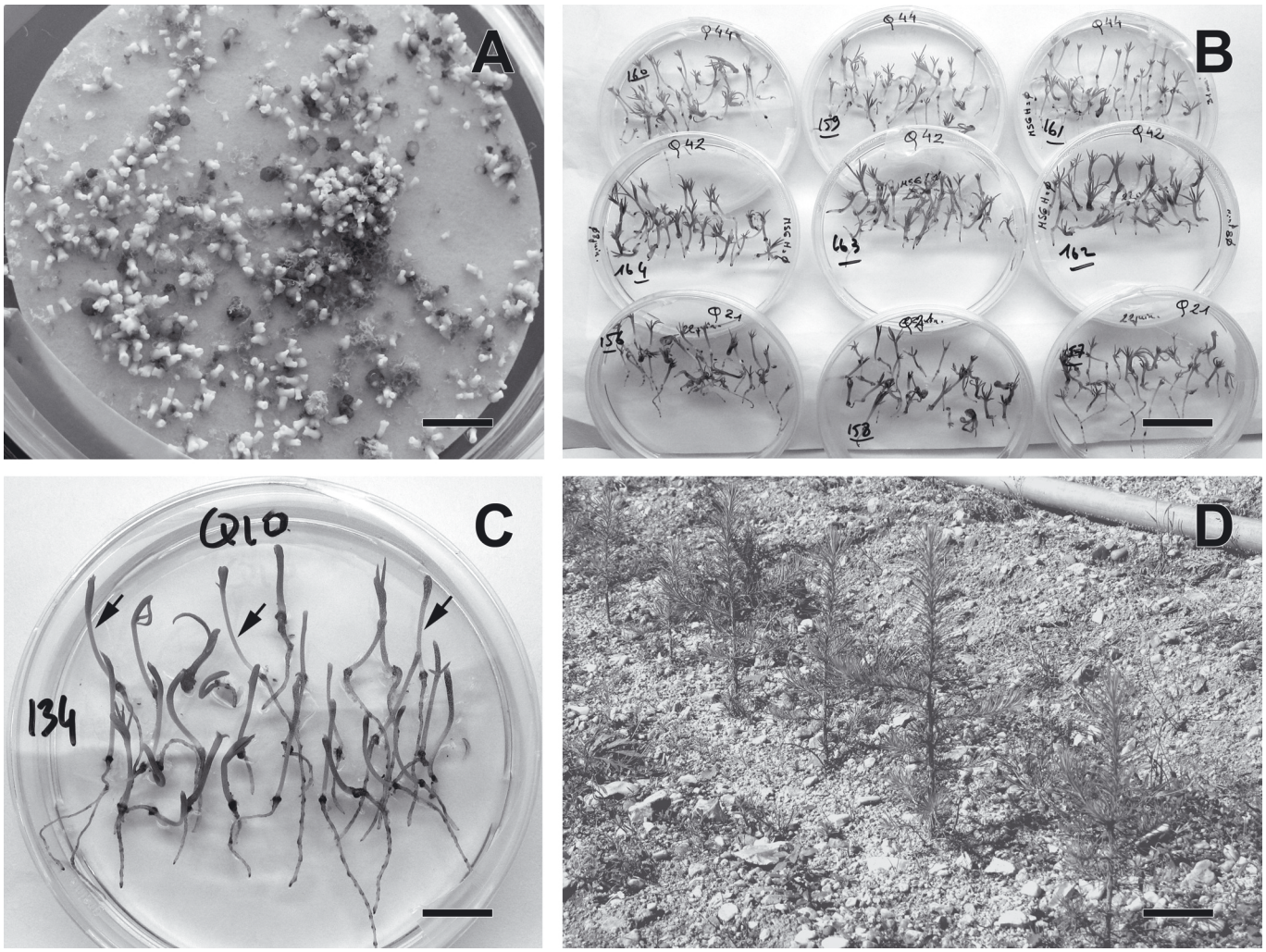

Figure 1. Somatic embryogenesis in hybrid larch (*Larix* × *eurolepis*): (A) Cotyledonary somatic embryos 5 weeks old developed in presence of gelrite 8 g·L^{-1}, line P21 bar = 9 mm (B) Germinated somatic embryos 2 weeks old, lines Q21, Q42 and Q44, bar = 3 cm (C) Detail of germinated somatic embryos, line Q10, notice elongated hypocotyls (arrows) bar = 1,2 cm (D) Plantlets transferred to soil 1 year old, bar = 5 cm.

embryos were well-shaped with nice elongated hypocotyls and cotyledons with reduced proliferation of EM that appeared dried (Fig. 1a). Besides a significant genotype effect, gellan gum concentration also had a significant influence on the number of somatic embryos produced (Fig. 2). Best results were obtained at the intermediate concentration (8 g·L^{-1}) of gellan gum. Nearly twice the number of embryos (464) on average was produced when compared with 4 g·L^{-1}gellan gum concentration (235). With 10 g·L^{-1}, results significantly decreased (371 embryos produced) compared to 8 g·L^{-1} (Fig. 2). Nevertheless, results varied according to lines as shown by the significant interaction effect (Tab. II).

3.5. Effect of age and cryopreservation on embryogenic potential (experiment 3)

The effect of ageing on maturation was tested for three REVE-VERT lines at age varying from 4 to 21 months. Po-

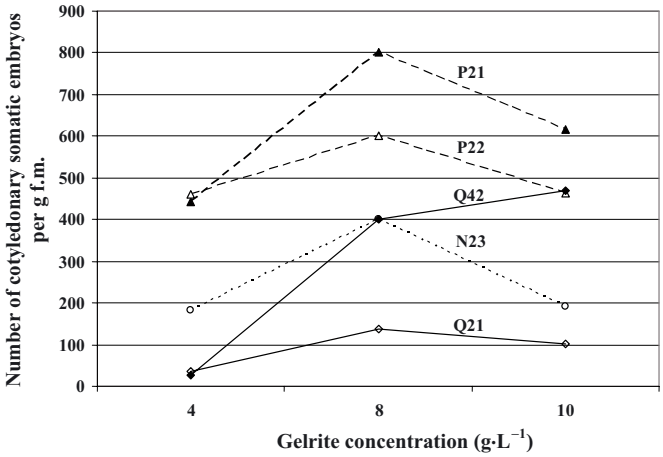

Figure 2. Variation of embryogenic potential of lines (*Larix* × *eurolepis*) according to gelrite concentration.

Table II. Embryogenic potential of lines of *Larix* × *eurolepis* according to gelrite concentration: ANOVA table.

	Ddf	MS	F
Gelrite	2	676411.4	118.5***
Line	4	1230394.3	215.5***
Interaction	8	98562.5	17.3***
Residual	124	5708.4	

tential for embryo production was high regardless of the age or genotype of the line (Tab. III). Ageing clearly did not reduce embryogenic potential. For one line (69-18), age effect could be tested even over a much longer period, extending from 60 up to 108 months. The number of embryos produced varied irregularly over time, from 1747 up to 2431 depending on age (Fig. 3). Age had a significant effect ($p < 0.009$) but the number of embryos produced at age 108 months was not significantly different from that produced at age 60 months.

Compared with the original line 69-18, somatic embryo production of the cryopreserved line 69-18c did not show any significant variation. On average 69-18 and 69-18c produced 930 ± 313 and 1171 ± 90 somatic embryos g^{-1} f.m., respectively. The cryopreservation per se and its duration (at least up to 3 years) had no effect on the recovery of embryogenic potential.

3.6. Maturation and plant production from hybrid larch *Larix* × *eurolepis* variety REVE-VERT (experiment 4)

Based on previous results, an optimised protocol for embryo maturation was defined and tested on 24 lines of REVE-VERT hybrid variety. This protocol involved maturation in darkness for 1 week on AC containing medium followed by 5 weeks on ABA medium containing gellan gum medium with a concentration of 8 g × L^{-1}. Among all the tested lines, one was not responding, i.e., no recovery of cotyledonary somatic embryo. For the others, on average 446 embryos per g of f.m. was obtained, but a high degree of variability was observed among lines. Differences among lines were significant ($p < 0.0001$). The best line produced up to 1566 embryos and the worse one only 8.2 embryos (Fig. 4). All the lines produced cotyledonary somatic embryos and regenerated plantlets. Germination for 1 week in darkness allowed elongation of the hypocotyl (Figs. 1b and 1c) that subsequently allowed an easy handling of the germinated somatic embryos. Increasing the gellan gum concentration of the maturation medium improved somatic embryo development. As shown in Table IV, the lowest concentration of gellan gum in the maturation medium (4 g·L^{-1}) significantly reduced embryo germination ($p < 0.05$) but there was no significant effect of concentration on further plantlet development (Tab. IV). Three months after acclimatization, plantlet survival in the shade house was high 84.6% ± 9.4 (301/354, mean ± CI 5%). After 8 months, 79.5% ± 9.5 (283/354, mean ± CI 5%) of the plantlets survived and were selected to be transferred to plain soil in the nursery where they are currently growing (Fig. 1d).

Table III. Effect of embryogenic line age on somatic embryo production in *Larix* × *eurolepis*.

Lines	Age (months)	Number of embryos produced	*p*-value
N23	7	748.8 ± 214.6	
	20	683.4 ± 285.7	0.348
P1	4	359.8 ± 25.6	
	11	422.2 ± 70.4	0.634
P22	9	590.3 ± 155.5	
	21	849.1 ± 108.2	0.015

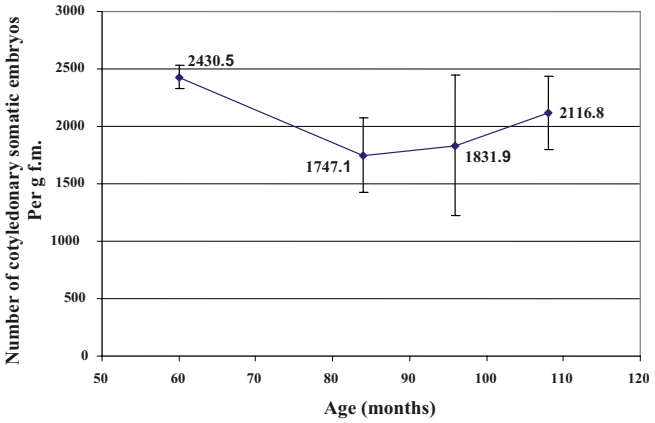

Figure 3. Effect of embryogenic line age on somatic embryo production (line 69-18, *Larix* × *marschlinsii*).

4. DISCUSSION

4.1. SE of hybrid larch: improvements and simplifications

In the current study we have optimised and simplified the protocols previously established (Klimaszewska, 1989; Lelu et al., 1994a) along the different phases of SE of hybrid larch. According to our results, an efficient and rapid method for production of large quantities of plants for clonal tests has been achieved. From the start of EM proliferation, the process takes about 3 months to regenerate acclimatized plantlets.

Considering the efficiency of the different steps of hybrid larch SE, efforts still need to focus on improving the initiation and proliferation phases (Fig. 5). It is well-known that the genotype influences SE. The initiation step is under the strong genetic control as reported for *Picea glauca* (Park et al., 1998), *Pinus taeda* (MacKay et al., 2006), *Pinus* species (Park et al., 2006). In *Larix* × *eurolepis*, initiation frequencies of EM (up to 65%, Fig. 5) were much higher than previously reported (25%, Klimaszewska, 1989). Indeed initiation could be improved by reconsidering either the formulation of the culture medium and PGRs (Park et al., 2006), or the cultural method by application of liquid medium (Pullmann and Skryabina, 2007). The search for optimized medium should continue. Culture of EM as a thin layer on filter paper resulted in a much more homogenous growth. As proliferation on filter paper disc yielded large quantity of EM, maturation experiments could be done quickly. This was very convenient to rapidly assess

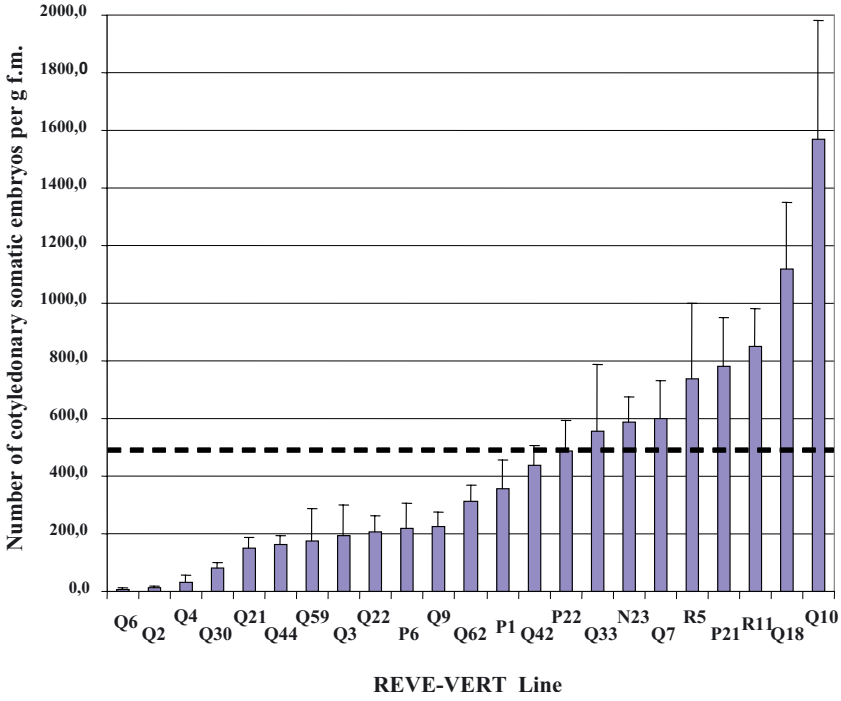

Figure 4. Mean number of cotyledonary somatic embryos produced per *Larix* × *eurolepis* line. Overall mean number of somatic embryos is represented by the dotted line.

Table IV. Effect of gellan gum concentration of the maturation medium on germination and plantlet production frequencies in *Larix* × *eurolepis*.

Gelrite g·L^{-1}	Germination frequency (%)	Plantlet frequency (%)
4	89,3 ± 6,6a	60,4 ± 11,1a
8	95,9± 2,9b	64,4 ± 7,1a
10	96,7 ± 3,6b	69,3 ± 8,61a

Mean ± CI 5%; different letters indicate significant differences among treatments ($p < 0.05$).

the embryogenic potential of the lines before their cryopreservation.

Somatic embryo production was dependent on the embryogenic line, a phenomenon also observed in other conifer species (Högberg et al., 1998; Lelu-Walter et al., 2006; Park, 2002). However, using the improved protocol, 94% of the lines produced mature somatic embryos (Fig. 5), which was above the level reported for hybrid larch (Klimaszewska, 1989; Lelu et al., 1994a). In general, maturation yields were also very high using the improved SE protocol. In comparison, Norway and black spruce cultures produced mature somatic embryos in 12 of 15 families and in 23 of 31 families, respectively (Adams et al., 1994; Högberg et al., 1998).

In hybrid larch, maturation of somatic embryos was greatly enhanced by first culturing EM sprayed over filter paper disc, method routinely used in *Pinus* species (Klimaszewska et al., 2007), then when ABA medium was solidified with 8 g·L^{-1} gellan gum. If the positive effect of high gellan gum concentration on somatic embryo maturation is well-known in pine species (Lelu-Walter et al., 2006; 2008), but had not been previously demonstrated with hybrid larches. Increasing the gellan gum concentration improved both the quantity and quality of the somatic embryos by reducing EM proliferation and giving rise to well-shaped somatic embryos which subsequently germinated and developed into plantlets.

Some simplifications were applied to initiation, cryopreservation and maturation steps. Previously, EM were initiated from immature zygotic embryos either co-cultured with half of the megagametophyte (Klimaszewska, 1989) or excised from the surrounding megagametophyte (Lelu et al., 1994a). These procedures are technically demanding and time consuming, therefore the zygotic embryos were cultured within the whole megagametophyte to simplify the excision proces. In larch, there is no cleavage polyembryony (Dogra, 1978; Owens, 1995). Only simple polyembryony occurs, i.e. fertilization of more than one egg cell per seed, which results in multiple genetically different zygotes. However, when multicellular embryo stage is reached in June, one embryo dominates while the others degenerate (Owens, 1995), hence most likely EM originated from a single zygotic embryo.

Maturation in darkness did not reduce the number or quality of somatic embryos. Maturation in darkness allows stacking more efficiently Petri dishes, saving considerable space. There is still a need to further simplify the process. The need for activated charcoal in the culture medium remains questionable. It has been proven to be efficient for maturation of *Picea* (Roberts et al., 1990) and more recently for maritime pine (Lelu-Walter et al., 2006). In hybrid larch, it has been demonstrated that AC adsorbed 90% of residual 2,4-D from

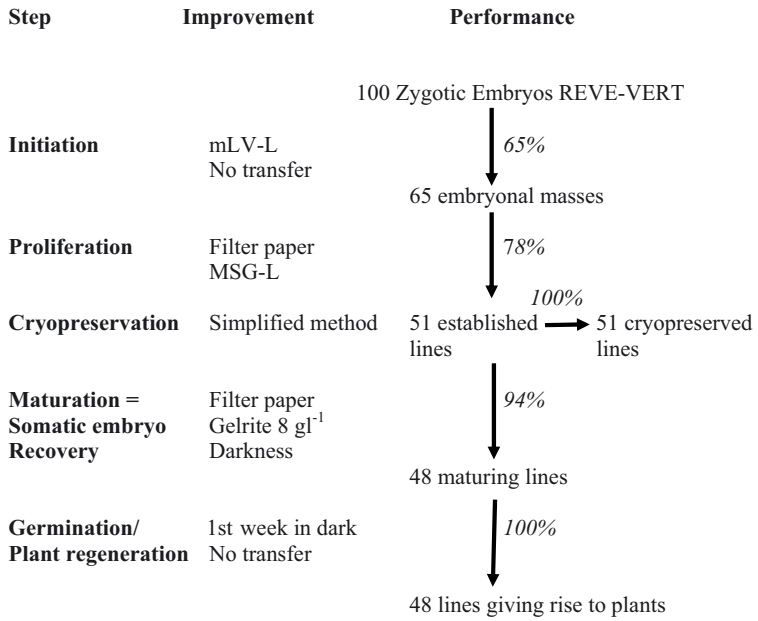

Figure 5. Summary of the improvements and performances of each step of hybrid larch somatic embryogenesis.

EM remaining from the proliferation medium (von Aderkas et al., 2002). However, the use of AC for 1 week did not improve the embryogenic potential (data not shown). There is always a compromise between the number and the quality of SE developed and the use of AC remains complex and still questionable.

4.2. SE of hybrid larch: further requirements

As shown in that study, the improved protocol was successfully applied to the new hybrid variety REVE-VERT. The high initiation rate that can be reliably obtained now and the successful maturation phase observed in that study represent major steps for the effective integration of SE into the larch breeding programme. As with any propagation system, breeders are concerned by the integrity of the SE-propagated improved variety both in terms of its performance and maintenance of its level of genetic diversity. Trees produced by SE must behave like or even better than seedlings in terms of growth, architecture, stability, maturation, etc. While performance validation in the field is still needed for larch, preliminary results on white spruce (Lamhamedi et al., 2000) and Norway spruce (Högberg et al., 2003) showed no or little differences for height at nursery stage in particular when best embryos were sorted out for lateral roots and/or epicotyl length.

Age of the embryogenic culture could influence the ability of EM to produce mature somatic embryos. This phenomenon is critical for species such as *P. pinaster* (Klimaszeswka et al., 2008). In hybrid larch ageing did not affect the maturation. A line can obviously maintain its embryogenic potential up to 9 years without a reduction. Cryopreservation of embryogenic lines overcame the age effect but genetic stability of the cryopreserved lines should be guaranteed on the long run (Park et al., 1998). Interestingly, in hybrid larch cryopreservation did not reduce embryogenic potential of EM (i.e. ability to regenerate somatic embryos).

Genetic diversity integrity includes also a second aspect. In the case of the REVE-VERT variety issued from a polymix with 12 male parents, the balanced contribution of the 12 male components should also be ascertained in final plant-lots. This aspect will be further investigated. Interestingly, SE in larch allows recovery of a much larger number of seed-lines per cone (65) than the actual available filled seed number (5) that can be recovered at maturity. Initiation of SE from immature seed occurs well-before abortion process leading to so many empty seed per cone at maturity. The interest of these additional seed-lines both in terms of their contribution to genetic diversity and to mean field performances should be investigated.

4.3. SE of hybrid larch: interests for the breeding programme

The efficiency of a tree breeding programme is linked to the ability to breed for and select high performing varieties for adaptive and commercial traits and to mass-propagate them. With hybrid larch, the latter has been so far problematic.

Classical hybridisation seed orchards suffer from low filled seed production together with variable, usually low, hybrid rate in seed lots (Fig. 6, way 1). In addition, establishment and management of a seed-orchard require a big investment which limits commercial exploitation for several decades. This slows breeding and selection progress from being rapidly transferred to the forest.

Classical vegetative propagation by cutting from hedged stock-plants proved also of limited use because of the quick

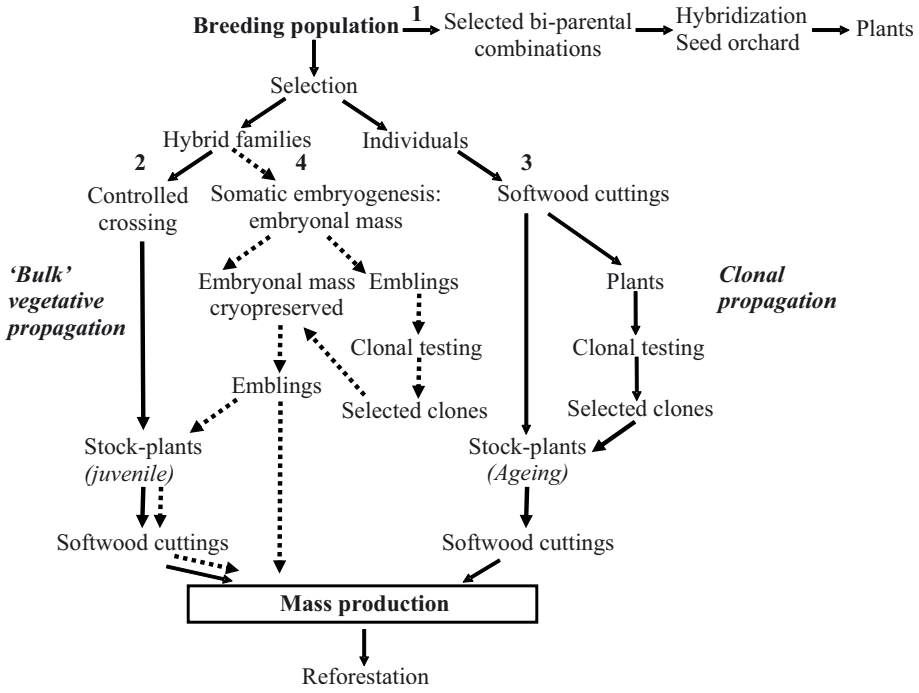

Figure 6. Somatic embryogenesis (dotted lines) as an alternative or a complementary way to mass-propagate hybrid larch varieties.

ageing of stock-plants, resulting in low rooting rates and plagiotropic growth habits (Fig. 6, way 3). With this scheme, when clones in clonal tests reach an adequate age for selection (10–15 years), stock-plants of selected clones are already physiologically too old for use in mass-production. An alternative, called 'bulk' vegetative propagation (Fig. 6, way 2), was tested using rooted cuttings from juvenile stock-plants (less than 4 years old from seed, Le Pichon et al., 2001). In that case, no clonal selection occurs and entire selected families (usually full-sib) or orchard progeny are propagated. That is the most promising technique so far: it is efficient in terms of rooting ability and quality of rooted plants and in addition, it allows producing 100% hybrid plants. However, from a breeder's point of view, this scenario does not allow taking benefit of the additional gain from individual selection as in true clonal propagation.

Among vegetative propagation techniques, SE, therefore, offers even further perspectives for breeders. It is directly linked to two conditions already recognised by Högberg et al. (1998) and Klimaszewska et al. (2007): the efficiency of the propagation system and the maintenance of juvenility via cryopreservation. Combination of SE together with cryopreservation will allow the additional clonal selection step missing in 'bulk' propagation. SE shows the potential to combine all advantages and avoid many disadvantages of previous propagation techniques. SE can result in large-scale production of plants of good quality, while simultaneously maintaining juvenility and production of 100% hybrid plants. This results in flexibility in breeding programme, including the balancing of genetic gain and genetic diversity in the management of the varieties in plantations.

Integration of SE into the hybrid larch breeding programme can also be considered as a step in the selection of a variety, which requires subsequent clonal testing (Fig. 6, way 4). Its use to recurrently generate clones for next generation as suggested by Klimaszewska et al. (2007) does not seem realistic so far, as long as composite hybrid breeding is not previously validated. Another use could be of course to use SE as an alternative to rooting cuttings to increase precision in progeny testing by vegetatively propagating hybrid full-sib families.

Mass-propagation of selected varieties by SE (Fig. 6, way 4) could be cost-effective when combine to cutting propagation (Fig. 6, way 2). Continuously juvenile stock-plants could be provided from cryopreserved lines allowing for efficient rooting both in quantity and quality.

For current hybrid larch breeding programme in France, more than one thousand full-sib and half-sib hybrid families have been created by controlled crosses and are being tested. Selection of the best families is underway for various combinations of traits. This will result in the best hybrid combinations being available for propagation via SE.

In conclusion, this hybrid larch (*L.* × *eurolepis*, *L. marschlinsii*) SE protocol should influence breeding strategies by offering an alternative tool for accelerated production of large quantities of plants for clonal tests. Based on the above results, we recommend the following:

– Rapid surface disinfection of the cones (no seed disinfection needed).
– Culture of immature zygotic embryos (within megagametophyte) on mLV-L medium without subculture during the whole initiation period.

– Proliferation of the EM on a filter paper disc to rapidly increase tissue fresh mass.
– Simplified cryopreservation method (no need for programmable freezer).
– Maturation in darkness on a filter paper disc placed first 1 week on PGR-free MSG medium followed by 5 weeks on MSG medium supplemented with 0.2 M sucrose, 60 μM ABA and 8 $g \cdot L^{-1}$ gellan gum. No subcultures are necessary during the entire maturation period.
– Germination of cotyledonary somatic embryos on PGR-free MSG medium, in darkness for the initial 7 days, followed by exposure to low light.
– Transfer of plants from the Petri dishes to the potting mix during vigorous growth phase, and acclimatization under shade house conditions.

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