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Abstract Several factors affecting somatic embryogenesis (SE) in *Pinus sylvestris* from self- and cross-pollinated seed families were studied with the aim of producing large quantities of clonal plants. SE initiation from zygotic embryos was improved on a medium with lower than standard concentrations of 2,4-dichlorophenoxyacetic acid (2.2 vs 9.5 µM) and 6-benzyladenine (2.2 vs 4.5 µM). On this medium, initiation rates of four controlled crosses, including one self-cross, varied from 3% to 25%. Among the maturation factors tested, the concentration of abscisic acid (ABA 80, 120 µM) had no significant effect on the production of mature somatic embryos when the medium contained 0.1M sucrose. When sucrose concentration was 0.2M, however, 1.4 times more mature somatic embryos were produced on medium with 80 µM compared with 120 µM ABA. Under our best maturation conditions, mature somatic embryos accumulated amounts of storage proteins that were similar to the amounts in mature zygotic embryos. Activated charcoal exerted a beneficial effect on mature somatic embryo production of 24-week-old cultures; there was no evidence of such an effect in 8-week-old cultures. Thirty seven embryogenic lines from a self-cross and an out-cross were chosen for clonal plant production. Highly embryogenic lines produced mature somatic embryos that were more likely to convert to plants than those from less embryogenic lines. After 4 months of growth in a shade house, plantlet survival rates exceeded 70% for 31 lines out of 35. This report describes an improved method for accelerated production of large quantities of Scots pine for clonal tests.

Abbreviations **ABA**, abscisic acid; **AC**, activated charcoal; **BA**, benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **DMSO**, dimethylsulphoxide; **EM**, embryonal mass; **f.m.**, fresh mass; **PGR**, plant growth regulator

Introduction

Somatic embryogenesis (SE) in conifers has become a powerful biotechnological tool for clonal plant production. As such, it has the potential of being applied in tree improvement programs and in research involving screening for disease resistance and other desirable traits because it may deliver any number of clonal individuals for either clone selection or pathogen and pest challenging tests.

Pinus sylvestris (Scots pine) has a wide natural distribution throughout much of Eurasia, and adaptation capabilities to diverse environments (Boratynski 1991). In France, gains in the genetic quality of Scots pine plantations are expected from improved breeding populations created from natural populations. Scots pine plantations at Haguenau, in eastern France, are appreciated for their superior height and diameter growth (Quencez and Bastien, 2000). In this context, our study was undertaken to improve existing protocols of *Pinus sylvestris* SE and apply these enhancements to embryogenic lines derived from immature zygotic embryos of the natural population of Haguenau for clonal plant production.

The first reports of SE in Scots pine focused mainly on initiation from immature seed, and investigated the responses of excised zygotic embryos at several developmental stages on various culture media (Keinonen-Mettälä et al. 1996; Lelu et al. 1999; Häggman et al. 1999). While regeneration of small numbers of somatic seedlings and young trees was achieved in those studies, efforts were not aimed specifically at developing somatic embryo maturation protocols for the efficient production of large numbers of clonal plants. Elsewhere, a diallel cross among seven parent trees was conducted to evaluate the effect of parent genotype on SE (Niskanen et al. 2004). There was a strong maternal effect on initiation of SE that was mostly independent of the paternal effect, a conclusion also reached in another study on *Pinus taeda* (MacKay et al. 2006).

In the present work, we focused on SE initiation from four controlled crosses, including one self cross, among three trees that had previously been tested for their response to initiation (Lelu et al. 1999). In other experiments, the effect of several factors on the maturation of somatic embryos was investigated. Those were culture age, abscisic acid concentration of the medium, its sucrose concentration and coating of the cells with activated charcoal (AC). The storage protein contents of cotyledonary somatic embryos of three distinct phenotypes, matured under the best of tested conditions, was measured and compared with that of zygotic embryos. Accumulation of

storage proteins could be used to assess the quality of somatic embryos and to determine optimal harvest time. Subsequently, the improved protocols were used to assess production of plants from embryogenic lines of an out-cross and a self-cross.

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Materials and methods

Plant materials

Pinus sylvestris (L.) trees 818 (A), 785 (B) and 666 (C) originated from eastern France (Haguenau). Open pollinated seeds of these trees were tested previously for their ability to initiate SE (Lelu et al. 1999). Tree A appeared the most responsive to initiation of SE with an initiation rate of 22% among seed explants, whereas trees B and C had initiation rates of 9% and 0%, respectively.

For the present study, the following controlled crosses were performed at INRA, Orléans, France, in 1999 and 2003: A x B, C x A, A x C and one self-cross, A x A. Immature cones were collected from mother trees twice: on 19 June 2000 and on 21 June 2004.

Initiation of SE and proliferation of embryonal mass (EM) (Experiments 1)

Prior to seed extraction, each cone was submerged in 95% (v/v) ethanol for 10 min, briefly dried in the laminar flow unit, and cut longitudinally into 2 pieces. Subsequently, scales with immature seeds were detached from the cone; 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%. Based on previously published results (Lelu et al. 1999), seeds were extracted when the zygotic embryos were at the cleavage polyembryony stage. At this early developmental stage, megagametophytes that contained zygotic embryos were carefully removed from the seed coat, nucellus and megaspore wall, and cultured intact on initiation medium. Explants were cultured in 90 x 20 mm Petri dishes, each of which contained approximately 30 ml of a semi-solid medium (Litvay et al. 1985) modified by reducing the concentration of macro elements by 50% (except iron and EDTA) and adding 1 gl^{-1} casein hydrolysate (enzymatic, SIGMA, CH), 0.5 gl^{-1} L-glutamine (SIGMA), 30 gl^{-1} sucrose, 2.4-dichlorophenoxyacetic acid (2,4-D) at either 9 or 2.2 µM, 6-benzyladenine (BA) at either 4.4 or 2.3 µM, and 4 gl^{-1} gellan gum (Phytagel™, SIGMA). The medium with the higher concentrations of 2,4-D and BA was designated as mLV-S (for standard concentration of plant growth

regulators, PGRs), and that with lower concentrations of the two compounds as mLV-L (for low PGRs). The medium without PGRs is subsequently referred to as the mLV medium.

The pH of each medium was adjusted to 5.8 after the addition of gellan gum. An appropriate aliquot of filter-sterilized stock solution of glutamine (also pH-adjusted to 5.8) was added to the medium after autoclaving. 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 SE if EM could be identified under the stereoscope. After 10 weeks, EM that showed continuous growth and produced amounts of fresh mass (f. m.) sufficient for subculture was considered to have "proliferated".

Some explants from crosses A x A and C x A initiated minute amounts of EM, but after 3 to 4 weeks, the tissue stopped growing. To "rescue" these lines and promote growth, the EM was suspended in a small volume of liquid medium and cultured on a filter paper disk (see below).

Experiment 1a: effect of PGR concentration

The effect of PGR concentration of the medium (low or standard) on the rate of EM initiation was assessed from explants of the A x B cross. The experiment was run in two blocks of 16 and 21 Petri dishes, respectively. The first block contained 8 Petri dishes for each medium, and the second, 11 Petri dishes with the standard PGR concentration medium, and 10 with the mLV-L medium. Petri dishes with initiated SE were used to assess the effect of PGRs on the rate of EM proliferation: in the first block, there were 3 such Petri dishes with mLV-S medium and 8 with mLV-L medium, and in the second, there were 10 Petri dishes from each PGR level.

Experiment 1b: effect of crosses

Rates of SE initiation by explants from four crosses (C x A, A x A, A x B and A x C) were compared on mLV-L medium. Explants from each cross were distributed in 11 to 17 Petri dishes for a total of 57 Petri dishes. Rates of EM proliferation, given initiation, could be assessed from 4 to 13 Petri dishes per cross.

First and subsequent subcultures of EM

The age of the embryogenic culture was set to 0 the day of the first subculture. Subsequently, the embryonal mass was subcultured every two weeks onto fresh mLV-Lmedium, except that the sucrose concentration was reduced to 20 gl^{-1}. Approximately 300 mg f.m. of proliferating EM was collected and suspended in 4 to 5 ml of liquid mLV-L medium, vigorously shaken to break up the tissue pieces into a fine suspension, and poured onto a filter paper (Whatman # 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, semi-solid mLV-L medium and cultured in darkness at approximately 25°C for 2 weeks. This procedure yielded 2 to 10 g of EM per filter paper disc depending on the line. All initiated embryogenic lines from crosses A x A and C x A stopped growing after reaching approximately 20 to 50 mg f.m. To promote growth of these lines, an explant with initiated EM was placed in a sterile Eppendorf tube with 0.5 to 1 ml of liquid mLV-L medium, shaken to break up the tissue clumps and poured onto a filter paper disc (Whatman # 2, diameter 7 cm) in a Büchner funnel. The filter paper with attached cells was placed on the surface of fresh, semi-solid mLV-L medium and cultured.

Cryopreservation protocol

The cryopreservation protocol used in this study has been previously published for *P. monticola* (Percy et al. 2000) and routinely applied since then to other conifer species (Lelu-Walter et al. 2006, K. Klimaszewska and M.-A. Lelu-Walter, unpublished). Briefly, 3 g f.m. embryogenic tissue cultured on filter paper for 7 d were suspended in 12 ml of liquid mLV-L medium supplemented with 0.4 M sorbitol for 18 h. Subsequently, 3 ml of dimethyl sulphoxide (DMSO, SIGMA) was added to the suspension on ice (final DMSO concentration 7.5%). After 1.5 h, 1 ml of suspension was transferred to a cryovial in a 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 culture recovery.

Seventeen lines, four from the A x B cross and 13 from the A x C cross, were frozen. Proliferation on filter papers yielded large quantities of EM and cryopreservation could be

carried out with lines as young as 2 weeks (since the first subculture). Until now, this cryopreservation technique has resulted in the recovery of all tested lines. Over the years, cryopreserved lines were routinely used in the experiments. Cryopreservation per se and its duration (up to 3 years) had no apparent effect on the yield of somatic embryos (data not shown). Experiments in which cryopreserved material was used are identified.

Factors influencing maturation of somatic embryos (Experiments 2)

A first set of maturation experiments were conducted with 7 lines initiated in 1999 (2 LS lines) and in 2000 (5 MS lines). Line LS4 originated from the A x C cross, and lines LS8, MS1, MS6, MS11, MS15 and MS17, from the A x B cross. The experiments were conducted between 2000 and 2004. They were designed to investigate the effects of various concentrations of abscisic acid (ABA, racemic, SIGMA) and sucrose in the maturation medium on the production of mature somatic embryos of one or several embryogenic lines sampled at one or more culture ages. Quantities of EM required in any experiment were collected approximately 1 week after subculture and suspended (by vigorous shaking) in liquid mLV medium with 0.08 M sucrose, without PGRs. Five ml of the suspension containing from 200 to 289 mg f.m. of EM were then poured onto a filter paper disc, as described above, and placed on mLV maturation medium containing 10 gl^{-1} of gellan gum, and ABA and sucrose in concentrations specific to each experiment. The ABA stock solution was filter sterilized and added to the molten medium after autoclaving. The medium was dispensed into 90 x 20-mm Petri dishes at approximately 40 ml per dish. Cultures were placed under a 16-h photoperiod, dim light (5 µmol m^{-2} s^{-1}) at approximately 24/21°C day/night temperature for 12 weeks, and were not subcultured during the course of the experiments. Productivity was measured as the number of morphologically normal mature somatic embryos per Petri dish produced over the 12-week period. Features specific to each experiment and exceptions to the general conditions above follow.

Experiment 2a: effect of ABA and sucrose concentrations

In the first of the maturation experiments, mean somatic embryo production of line MS6 on maturation media containing either of four combinations of ABA (80 or 120 µM) and sucrose

(0.1M or 0.2M) concentrations was compared. There were 3 to 6 Petri dishes per ABA and sucrose concentrations, for a total of 17.

Experiment 2b: effect of ABA concentration combined with 0.1M sucrose

The effect of the two ABA concentrations of the maturation medium, 80 and 120 µM, on somatic embryo production was also assessed for embryogenic tissue of lines MS1, MS6, MS11 and MS15 from the A x B cross. The embryogenic cultures were about 4 weeks old, and the sucrose concentration of the maturation medium was 0.1M. There were 2 or 3 Petri dishes per line, per ABA concentration for a total of 22.

Experiment 2c: effect of ABA concentration combined with 0.2M sucrose

The same ABA concentrations of the maturation medium, 80 and 120 µM, were tested with embryogenic tissue of line LS4 from the A x C cross, and lines LS8, MS6, MS15 and MS17 from cross A x B. Culture ages of the embryogenic tissue, which was retrieved from cryopreservation, varied from 6 to 14 weeks. The maturation medium contained 0.2M sucrose. The number of Petri dishes per line, per ABA level varied from 3 to 10 for a total of 108 Petri dishes.

Experiment 2d: effect of activated charcoal (AC)

In another experiment, half the EM was suspended in liquid mLV medium containing AC (Merck, at 10 gl^{-1}). The cells were then cultured on maturation medium containing ABA (80 µM), sucrose (0.2M) and gellan gum (10 gl^{-1}), as previously described. When the liquid suspension medium contained AC, both cells and AC particles coating the cell aggregates were collected on the filter paper. The AC effect was tested on three lines: LS4 from the A x C cross, and MS6 and LS8 from cross A x B. Cultures were either 8 or 24 weeks old at the onset of this maturation experiment and were retrieved from cryopreservation. There were 4 to 10 Petri dishes per line, per AC level (0 or 10 gl^{-1}), per culture age, for a total 86 Petri dishes.

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Storage protein extraction and quantification in zygotic and somatic embryos

Cotyledonary somatic embryos of line MS6 (A x B cross) that developed on medium containing 0.2M sucrose, 80 µM ABA and 10 gl^{-1} gellan gum were collected for storage protein quantification. After 8 weeks of maturation, somatic embryos reached the cotyledonary stage and appeared totally white. After 10 to 12 weeks of maturation there were distinct differences among the cotyledonary somatic embryos with respect to their color. Some had yellow cotyledons and white hypocotyl, and others, green cotyledons with yellowish hypocotyl. To determine if the different phenotypes were associated with varying levels of storage reserves, total proteins were extracted and quantified. Their quantities were compared with those of mature zygotic embryos harvested at the end of November. Methods of total protein extraction, electrophoresis and quantification in zygotic and somatic embryos were identical to those described in Klimaszewska et al. (2004). Briefly, total proteins were extracted from 15 mg f.m. frozen embryos in 500 µl Tris-HCl buffer (pH 6.8) containing 2% SDS (w/v) and 28% (v/v) glycerol, and then heated to 95°C for 15 min. To reduce the samples, β-Mercaptoethanol was added to the extraction buffer at 5% (v/v) prior to heating. The extracts were then centrifuged at 15 000 g for 5-10 min and the supernatants were collected. Protein concentration in each sample extract was determined using the Bradford protein assay (Bio-Rad Protein Assay kit; Bio-Rad, Hercules, CA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following standard protocols. After SDS-PAGE the gels were stained with Coomassie blue R-250. Protein bands were quantified with the SYNGENE Bio Imaging System (Frederick, MD) in two gels.

Plant production from lines of crosses A x A and A x B (Experiments 3)

Experiment 3a: maturation of somatic embryos

Based on the previous experiments, mLV medium containing 0.2M sucrose, 80 µM ABA and 10 gl^{-1} gellan gum was selected to promote somatic embryo development in 35 lines (4 lines from A x A and 31 lines from A x B). Culture age was about 19 weeks, and there were 3 to 8 Petri dishes per line for a total of 189 Petri dishes. Productivity was assessed as in Experiments 2.

Experiment 3b: conversion of somatic embryos to plants

Mature, morphologically normal, white somatic embryos from 37 lines (5 lines from A x A and 32 lines from A x B), including those tested in Experiment 3a, were picked from Petri dishes after 12 weeks of maturation and placed horizontally, all in the same orientation, on the surface of mLV medium without PGRs, with 30 gl^{-1} sucrose and 4 gl^{-1} gellan gum to promote germination and conversion to plants. In this germination phase, there were 7 to 32 somatic embryos per Petri dish (90 x 20 mm), usually 15, 20 or 25, and a total of 158 Petri dishes, each containing 30 ml of medium. The Petri dishes were tilted vertically at an angle of approximately 35° to 40° and placed in darkness for 10 to 14 days at day/night temperatures of 24/21°C to promote hypocotyl elongation and reduce anthocyanin accumulation. Somatic embryos were then exposed to a 16-h photoperiod (10 µmol m^{-2} s^{-1}) at 24/21°C day/night temperatures. The plantlets were subcultured once onto fresh medium of the same composition after 6 to 7 weeks. The number of somatic embryos that germinated and converted to a plant after 16 weeks of germination was noted for each Petri dish.

Experiment 3c: acclimatization of somatic plants

For further plant production, 30 to 40 somatic plantlets from 35 lines (4 lines from A x A and 31 lines from A x B) were transferred from Petri dishes to a potting mix composed of peat moss (75%), vermiculite (25%) and slow release fertilizer osmocote (750 gm^{-3}) in trays. The trays were placed directly in a shade house at INRA, Orléans, France, in mid May 2005. During the first 2 to 3 weeks, a misted plastic sheet covered the plantlets to maintain high humidity. It was then lifted progressively. Plants were watered as needed. After 1 month, the somatic plants were treated as seedlings of a similar age. The survival of each plant was assessed 4 months after transfer to the shade house. In March 2006, 10 months after acclimatization, plants were selected and transferred to soil in the nursery.

Statistical Analysis

Most statistical models were of the generalized linear type (Agresti 2002, Chapters 4-6; Mize et

al. 1999). When the response variable was a number of "successes" (initiation, proliferation, conversion to plantlet, or plant survival after 4 months) out of a number of "trials" (explants, or plantlets), it was assumed to follow a binomial distribution. When it was a count, the number of somatic embryos produced from EM in a Petri dish, it was assumed to follow a Poisson distribution. Unless otherwise stated, models included an over-dispersion parameter, which accounted for variation among Petri dishes within medium, cross or line. It was estimated by Pearson's method. Means and their 95% confidence limits were computed on a transformed scale, the logit for binomial rates, and natural logarithm for Poisson counts, and back-transformed for presentation in the tables or in the text. In the latter, pairs of numbers in parentheses that follow a mean rate, a mean count or a ratio of mean counts are its 95 % confidence limits (C.L.). Models for counts included initial fresh mass (f.m.) of EM per Petri dish as an offset variable so that the mean number of somatic embryos is expressed on the basis of 1 g f.m. of EM. Equality between any two mean binomial rates was assessed by testing the equivalent null hypothesis that the difference between their logits was zero. A priori contrasts were the preferred method of comparison among means. All statistical tests were performed at the $\alpha = 0.05$ level. Observed p-values are denoted by lower case p, and degrees of freedom associated with F or chi-squared (χ^2) statistics, by d.f. Analyses were performed with the GENMOD procedure of SAS software (Version 9.1, SAS Institute Inc., NC; see also Littell et al. 2002, Chapter 10).

Two specific models are detailed below as examples: one for the SE initiation rate of Experiment 1b, and the other for the somatic embryo count of Experiment 2d. Specific models can be inferred from the information provided in the tables. Consider the logit model for the rate of SE initiation among explants in Experiment 1b. Let P_{Ii} denote the expected rate of initiation in Petri dish j ($j = 1, \ldots, n_i$ where n_i is the number of Petri dishes from cross i, i = 1, 2, 3, 4 for crosses A x C, A x B, C x A and A x A, respectively) containing m_{ij} explants from cross i. It was assumed that r_{ij}, the observed number of explants that initiated SE in this Petri dish, followed a binomial distribution, and that the logit of P_{Ii} depended linearly on four parameters:

$$\mathrm{logit}\left(P_{Ii}\right) = \log\left(\frac{P_{Ii}}{100 - P_{Ii}}\right) = \mu + \tau_i \qquad [1]$$

where μ is a reference parameter corresponding to the self cross A x A (i = 4), τ_i is the difference between the logit(P_{Ii}) for cross i (i = 1, 2, 3) and that for cross A x A, and $\tau_4 = 0$. A priori

contrasts between the initiation rates of the four crosses were also performed on that scale. For example, equality of P_{I4} for the self and P_{I3} for the C x A cross was tested as H_0: $\tau_3 = \tau_4$ vs H_1: $\tau_3 \neq \tau_4$, which is equivalent.

The analysis of EM proliferation rates in Experiment 1b was based on a model much like [1] except for the definition of P_{Ii} which became the expected proliferation rates for cross i, P_{Pi}, given initiation of SE. In Experiment 1a, the initiation and proliferation rates were also analyzed with a model like [1], but the τ_i's were the effects of the two PGR levels, low and standard, i = 1, 2, with $\tau_2 = 0$. The rate of conversion to plant in Experiment 3b was also based on models similar to [1] with τ_i's (i = 1, …, 37) representing line effects. A contrast between the τ_i's was constructed to compare lines from the self- and out-cross. In Experiment 3c, the overdispersion parameter of the model for the survival rate after 4 months was set to 1 since plantlets were treated individually.

Experiment 2d provides an example with counts of somatic embryos. It involved three factors: AC (i = 1 for presence, i = 2 for absence), line (j = 1, 2, 3 for lines LS4, LS8 and MS6, respectively) and culture age (k = 1 for 8 weeks, and k = 2 for 24 weeks). It was assumed that y_{ijkl}, the number of somatic embryos counted in the l^{th} Petri dish with the i^{th} AC level, j^{th} line and k^{th} culture age (l = 1, …, n_{ijk}, and n_{ijk} is the number of Petri dishes per combination of AC level, line and culture age) followed a Poisson distribution with mean μ_{ijk}. The model was:

$$\log\left(\mu_{ijk} \mid x_{ijkl}\right) = \mu + \tau_i + \lambda_j + (\tau\lambda)_{ij} + \alpha_k + (\tau\alpha)_{ik} + (\lambda\alpha)_{jk} + (\tau\lambda\alpha)_{ijk} \qquad [2]$$

where μ_{ijk} is the expected embryo count g^{-1} f.m. in the l^{th} Petri dish at levels i, j and k of the AC, line and age factors, respectively, x_{ijkl} is the embryogenic mass deposited on that Petri dish, μ is a reference parameter corresponding to absence of AC (i = 2), line MS6 (j = 3) and 24-week-old cultures (k = 2), τ_i is the effect of level i of AC ($\tau_2 = 0$), λ_j is the effect of line j ($\lambda_3 = 0$), α_k is the effect of culture age k ($\alpha_2 = 0$), and $(\tau\lambda)_{ij}$, $(\tau\alpha)_{ik}$, $(\lambda\alpha)_{jk}$, and $(\tau\lambda\alpha)_{ijk}$ are interaction effects between AC levels, lines and culture ages. The μ_{ijk}'s at various combinations of the factor levels were compared on the logarithmic scale through a priori contrasts between the τ_i's, the λ_j's, the α_k's or the interaction parameters of model [2]. Similar, but simpler models were constructed to analyze counts of somatic embryos in Experiments 2a, 2b, 2c and 3a.

Results

SE initiation and EM proliferation (Experiments 1)

SE initiation did not guarantee continued proliferation of EM. In the self cross A x A and the outcross C x A, the initiated EM did not proliferate unless cultured on a filter paper (see Materials and Methods section). Proliferation of some lines from these crosses was the result of this specific culture technique (Experiment 1b). For the A x A cross, six lines were rescued out of 13 initiated, and for the C x A cross, five lines out of 17 initiated. Generally, SE initiation required 5 to 7 weeks, after which the tissue was subcultured and proliferated further.

Experiment 1a: PGR concentration

In Experiment 1a, explants were more likely to initiate SE on mLV-L medium than on mLV-S medium ($p = 0.0002$, Table 1). However, EM proliferation rates did not appear to differ between the two media ($p = 0.25$).

Experiment 1b: Initiation and proliferation rates of four controlled crosses

Initiation rates differed among the four crosses tested in Experiment 1b ($p \leq 0.0001$, Table 2). On average, they were higher for the out-cross of mother tree A with father trees B and C than for the self cross A x A ($p \leq 0.0001$). They did not differ significantly between the two out-crosses of mother tree A ($p = 0.47$). The initiation rate of explants from these two out-crosses averaged $P_{I,\ AxB\ or\ AxC} = 22\%$ (C.L.: 17%, 29%). There was no indication that initiation rates differed between the out-cross of father tree A with mother tree C and the self-cross ($p = 0.35$).

Given initiation, proliferation rates of EM differed somewhat among crosses ($p = 0.03$, Table 2). The mean proliferation rates of the self cross A x A and the out-cross C x A did not differ significantly, nor did those of the two out-crosses of mother tree A with father trees B and C which averaged $P_{P,\ AxB\ or\ AxC} = 89\%$ (C.L.: 77%, 95%), but the proliferation rate of the self-cross was significantly lower than that of the two out-crosses of mother tree A.

Factors influencing maturation of somatic embryos (Experiments 2)

Experiment 2a: effect of ABA and sucrose concentrations

There was no indication that either ABA or sucrose concentrations or their interaction had any effect on somatic embryo production of line MS6 ($p = 0.81$, 0.22 and 0.57 for the three effects, respectively).

Experiments 2b and 2c: effect of ABA concentration combined with either 0.1M or 0.2M sucrose

In Experiment 2b, where the sucrose concentration of the maturation medium was 0.1M, the effect of the ABA concentration was tested with four embryogenic lines from the highly productive A x B cross. There was no indication that average somatic embryo production differed between media with 80 and 120 µM of ABA ($p = 0.68$), nor that the effect of ABA concentration differed among the lines tested ($p = 0.46$ for the ABA x Lines interaction). Somatic embryo production varied significantly among lines from an average of 29 g^{-1} f.m. of EM for line MS11 to 473 for line MS6 ($p = 0.0005$ for the main effect of lines). Productivity of line MS6 was 7.2 times (C.L.: 3.2, 16.0) as abundant as that of the three other lines, on average, but it did not differ among the latter three lines.

Experiment 2c was similar to Experiment 2b except that the maturation medium contained a higher sucrose concentration, and most lines differed (Table 3). Somatic embryo production was 1.4 times (C.L.: 1.1, 1.9) more abundant when the medium contained 80 µM ABA than when it contained 120 µM ($p = 0.014$ for the main effect of ABA). There was no indication that this ABA effect varied among lines ($p = 0.19$ for the ABA x Lines interaction), but average production varied considerably among lines ($p \leq 0.0001$): from 57 somatic embryos g^{-1} f.m. for line MS17 to 441 for line LS4. In particular, line LS4 from the A x C cross produced 2.6 times (C.L.: 1.4, 1.6) more somatic embryos than lines from the A x B cross ($p \leq 0.0001$), on average. The latter lines also differed in their productivity; one of them, MS6, produced almost as many somatic embryos as the A x C line, LS4.

Experiment 2d: effect of AC

Results from Experiment 2d suggested that the effect of AC on somatic embryo production depended on culture age at the start of maturation ($p \leq 0.0001$ for the AC x Age interaction, Table 4), as well as on the embryogenic line ($p = 0.006$ for the AC x Lines interaction). There was no indication that the effect of the AC x Age interaction varied among lines, however ($p = 0.18$ for the AC x Age x Line interaction), or equivalently, that the effect of the interaction between AC and the lines changed with culture age. Culture of EM in the presence of AC enhanced average somatic embryo production of line MS6 about 2-fold (the ratio of the two mean counts is 2.08, $p \leq 0.0001$) and that of line LS4 by a factor of 1.4 ($p = 0.02$), but had no apparent effect on the yield of line LS8 ($p = 0.39$). Average somatic embryo production from 8-week-old cultures did not seem affected by AC ($p = 0.36$), whereas production increased 2.10-fold when AC was present in 24-week-old cultures ($p \leq 0.0001$). The effect of culture age varied among lines ($p = 0.003$ for the Lines x Age interaction): average productivity of the three lines was higher in 8-week-old cultures than in 24-week-old ones ($p \leq 0.0001$ for the main effect of age), but the size of this effect varied from a 2.2-fold improvement for line LS8 to a 4.4-fold increase for line LS4.

Quantification of storage proteins

The electrophoretic separation of the total protein extracts under non-reducing conditions (Fig. 2A) showed the following proteins to be the most abundant: 55.0, 44.8, 37.2, 35.9, 33.3, 21.5, 16.7 and 14.2 kD, whereas under reducing conditions (Fig. 2B) the proteins were: 44.8, 36.3, 22.0, 11.5 kD. The 55.0 kD protein complex (a) dissociated into 35.5 – 36.8 (a1) and 22 – 22.4 (a2) polypeptide sets and represented the buffer-insoluble fraction of 11S globulins legumin-like (Bewley and Black 1985, Gifford 1988, Klimaszewska et al. 2004, Brownfield et al. 2007). The 44.8 kD complex did not dissociate and represented the buffer-soluble fraction of the proteins, 7S globulins vicilin-like (Gifford 1988, Klimaszewska et al. 2004, Brownfield et al. 2007).

Quantification of the polypeptide sets in cotyledonary stage, mature zygotic embryos as well as in cotyledonary-stage somatic embryos displaying distinct phenotypes showed differences (Table

5). Among the three phenotypes, white somatic embryos had the highest amounts of storage proteins, followed by yellow and green phenotypes. Overall, the somatic embryos of white phenotype accumulated only 4% less buffer-soluble proteins and 20% less buffer-insoluble proteins than mature zygotic embryos. Somatic embryos of the green phenotype had 40% less buffer-soluble proteins and 55% less buffer- insoluble proteins than zygotic embryos. Somatic embryos of the yellow phenotype had an intermediate level of storage protein accumulation. Germination of green somatic embryos was the most rapid because after one week of culture on the germination medium 92% of them developed ~0.5 cm radices followed by 75% and 56% of the yellow and white somatic embryos, respectively. However, final germination rates (scored after 6 weeks) for somatic embryos of all phenotypes were over 90%.

Plant production (Experiments 3)

Experiment 3a: maturation of somatic embryos for plant production from newly initiated lines of crosses A x A and A x B

Average somatic embryo production of lines from cross A x B was 4.8 times (C.L.: 2.4, 9.5) as abundant as that of lines from the self cross A x A (averages per cross: 167 (C.L.: 156, 180) and 35 (C.L.: 18, 69) somatic embryos g^{-1} f.m., respectively; $F = 68.0$ with 1 and 154 d.f.; $p \leq 0.0001$). Mean somatic embryo production varied among lines within crosses: from 3 (C.L.: 0.3, 38) to 233 (C.L.: 168, 324) g^{-1} f.m. among the four lines from the self-cross, and from 28 (C.L.: 11, 73) to 681 (C.L.: 559, 828) g^{-1} f.m. among those from the out-cross ($F = 21.2$ with 33 and 154 d.f.; $p \leq 0.0001$).

Experiment 3b: germination and conversion of somatic embryos to plants

Somatic embryos from lines of the out-cross A x B were more likely to develop into plantlets than those from lines of the self cross A x A ($F = 46.0$ with 1 and 121 d.f.; $p \leq 0.0001$). The average rate of conversion to plant for the out-cross lines was 62% (C.L.: 58%, 65%) and that for the self-cross lines, 28% (C.L.: 19%, 39%). The rate of conversion to plant differed among lines within crosses ($F = 3.74$ with 35 and 121 d.f.; $p \leq 0.0001$). Most lines from the self cross had

conversion rates smaller than or equal to 38% (PS1, PS2, PS3 and PS6), but somatic embryos of line PS4 from the self-cross had a rate of conversion to plant of 62% (C.L.: 47%, 74%), equal to the average for the out-cross lines. Somatic embryos from 7 out of 37 lines had rates of conversion to plantlets of 75% or more. For 34 lines that were common to Experiments 3a and b, the rank correlation between average somatic embryo production and rate of conversion to plant was 0.65.

Experiment 3c: acclimatization in a shade house and plant survival

Somatic plants transferred to a potting mix continued to grow under ambient conditions in the shade house. After 4 months, the mean survival rate of plantlets was 73% (C.L.: 59%, 83%) among the four lines of the self-cross, and 81% (C.L.: 78%, 84%) for 31 lines of the A x B out-cross. The difference was not significant ($\chi^2 = 1.68$ with 1 d.f.; $p = 0.19$). There was some indication that survival rates varied among lines within crosses ($\chi^2 = 54.05$ with 33 d.f.; $p = 0.01$). In March 2006, selected plants were transferred to soil in the nursery where they are currently growing with seedlings.

Discussion

In this study, tree A was used as the female parent in three of the four crosses including a self cross. SE initiation rates clearly indicated that tree A was the best when used as the female parent with trees B and C. However, when used as the male parent with tree C and in selfing, SE initiation rates were considerably lower. For crosses C x A and A x A, they were 5% and 3%, respectively. The EM of the latter two crosses was difficult to proliferate and survived owing to the special culture technique applied at the onset of the first subculture and afterwards.

The genetic control of SE initiation has already been well documented for various conifer species such as *Picea glauca* (Park et al. 1993), *Pinus sylvestris* (Niskanen et al. 2004) and *P. taeda* (MacKay et al. 2006). In all these studies, the maternal effect was very strong relative to paternal and other effects. The latter report also proposed a strategy to capture large numbers of embryogenic lines per seed family when targeting a small number of full-sib families. The approach was based on evaluating open-pollinated seeds of maternal and paternal trees intended for controlled crosses, and then using seed families from crosses that involved the best SE initiator as maternal parent. Based on our results and those of Häggman et al. (1999), Lelu et al. (1999) and Niskanen et al. (2004), the same approach and breeding strategy would be applicable to Scots pine.

An important result was that the embryogenic lines derived from selfed seeds of tree A proliferated and produced somatic plants. To maintain vigorous growth of the tissue, it was necessary to culture the cells spread on the surface of a filter paper placed on the semi-solid medium. Subsequently, the cultures produced mature somatic embryos and plants, which are presently tested in the nursery. To our knowledge, this is the first demonstration of clonal plant production from a selfed seed family of Scots pine. In the study of Niskanen et al. (2004), two embryogenic lines derived from two selfed seed families proliferated, but no plant regeneration was reported.

The explant response of Scots pine to SE initiation indicated that the medium with low concentrations of PGRs was more beneficial than the medium with standard concentrations. Hence, all further experiments were carried out on mLV-L medium, which was also used for proliferation of EM. These results were similar to the responses of *Pinus monticola* (Percy et al. 2000) and *P. strobus* (Klimaszewska et al. 2001), but were opposite to the responses of *P.*

pinaster (Lelu-Walter et al. 2006), which performed better on a medium with standard concentrations of PGRs.

ABA concentration had a significant effect on productivity of mature somatic embryos only when tested with 0.2M sucrose, in which case EM produced 1.4 times more somatic embryos on medium with 80 µM ABA than on medium with 120 µM ABA. AC was beneficial to older cultures (24-weeks old), but did not seem to affect embryo yield of younger ones (8-weeks old). In a similar study of somatic embryo maturation of *P. pinaster*, charcoal enhanced productivity, irrespective of culture age (Lelu-Walter et al. 2006). It is conceivable that in Scots pine, older embryogenic tissues internalized more PGRs than young ones during the prolonged culture, and that activated charcoal adsorbed some of the PGRs from the cells, reducing their residual effect (Ebert et al. 1993; von Aderkas et al. 2002).

Accumulation level of storage proteins differed among the three phenotypes of cotyledonary somatic embryos. These phenotypes became conspicuous after 10 to 12 weeks of maturation. The green somatic embryos had the lowest contents of buffer-soluble and buffer-insoluble storage proteins compared with those of the white phenotype and zygotic embryos. The green somatic embryos germinated most rapidly after transfer to a germination medium and taken together with the lowest amount of storage proteins indicated that the process might have already started on the maturation medium. White cotyledonary somatic embryos accumulated the highest protein amounts that were close to the amounts in mature zygotic embryos (96% of buffer-soluble and 80% of buffer-insoluble storage proteins). These results indicated that there was some asynchrony in the maturation process of somatic embryos and those which matured sooner started germinating while still on the maturation medium. Similar phenomenon was observed in *P. strobus* somatic embryos where extension of the maturation period resulted in diminished levels of storage proteins caused, most likely, by the onset of germination (Klimaszewska et al. 2004). Therefore harvesting time of the somatic embryos must be carefully determined if biochemical similarity with zygotic embryos is required.

Production of somatic embryos that have biochemical similarity with zygotic embryos is considered a benchmark of embryo quality and a vigorous post-germinative growth (Pullman et al. 2003). However, this has not been achieved yet in any conifer species. For example, *Pinus strobus* mature somatic embryos had quantities of storage proteins that were only 50 and 30% of the buffer-soluble and buffer-insoluble fractions, in their zygotic counterparts, respectively

(Klimaszewska et al. 2004). Similarly lower amounts were also found in mature somatic embryos of interior spruce (Cyr et al. 1991) and white spruce (Misra et al. 1993) indicating the need for further improvements in the maturation treatments for those species. However, recent study in loblolly pine showed that somatic embryos produced less triacylglycerol but more storage protein overall than zygotic embryos (Brownfield et al. 2007). This protein was predominantly buffer-soluble and amounted to a higher ratio of soluble to insoluble proteins compared with zygotic embryos suggesting possible difference in metabolic activity at the time of desiccation.

The somatic embryo maturation yield was strongly dependent on the embryogenic line, a phenomenon common to all conifer species. It is noteworthy that there was a correlation between somatic embryo production and rate of conversion to plant. The relatively high, positive rank correlation indicated that lines that produced a lot of somatic embryos were likely to have a higher rate of conversion to plant than lines that produced few somatic embryos. After 4 months of growth in the shade house, average survival rates did not differ between A x A and A x B plants. Six months later, the clonal plants were selected for transfer to a nursery for further growth. Plants were transferred to the field for clonal test in the spring of 2007.

In conclusion, this *P. sylvestris* SE protocol offers a relatively simple method for accelerated production of large quantities of plants for clonal tests. Based on the above results, we recommend the following:

- Surface disinfection of cones, no disinfection of seed is necessary
- Excision and culture of megagametophytes, with zygotic embryos, at the stage of cleavage polyembryony on mLV-L medium without subculture during the whole initiation period
- Proliferation of the embryonal mass on a filter paper for a rapid increase in tissue fresh mass.
- Simplified cryopreservation method (compared with the published one by Häggman et al. 1998) without the use of programmable freezer.
- Maturation of 200 mg f.m. on a filter paper placed on mLV medium containing 0.2M sucrose, 80 µM ABA and 10 gl^{-1} gellan gum for up to 12 weeks, no subcultures are necessary during the entire maturation period

- Germination of mature somatic embryos on the surface of mLV medium, in darkness, for the initial 7 to 10 days, followed by exposure to light. Petri dishes should be tilted for the entire period.
- Transfer of plants from the Petri dishes to the potting mix during vigorous growth phase, and acclimatization under shade house conditions (with initial maintenance of high relative humidity) commencing at the appropriate date specific to the climatic region. Elimination of the need for a greenhouse.

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Table 1 Experiment 1a. **A**) Rates of SE initiation (P_I, %) and rates of EM proliferation (P_P, %) of *P. sylvestris* on mLV medium with either of two concentrations of PGRs, followed by their 95% confidence limits (Lower, Upper), and **B**) Analysis including numerator (Num.) and denominator (Den.) degrees of freedom (D.f.), F statistics (F), and their p-values (p)

A) Rates

Medium	P_I	95 % confidence limits		P_P	95 % confidence limits	
		Lower	Upper		Lower	Upper
mLV-L	24	18	30	59	41	75
mLV-S	9	06	14	40	18	68

B) Analysis

Source of variation	Num. D.f.	Initiation			Proliferation		
		Den. D.f.	F	p	Den. D.f.	F	p
PGR	1	34	16.78	0.0002	28	1.39	0.25

Table 2 Experiment 1b: **A**) Rates of SE initiation (P_I, %) and rates of EM proliferation (P_P, %) on mLV-L medium for seed families of four crosses among *P. sylvestris* trees 818 (A), 785 (B) and 666 (C) followed by their 95% confidence limits (Lower, Upper), and **B**) Analysis including numerator (Num.) and denominator (Den.) degrees of freedom (D.f.), F statistics (F), and their p-values (p)

A) Rates

Mother	Father	P_I	95% confidence limits		P_P	95% confidence limits	
			Lower	Upper		Lower	Upper
C	A	5	3	11	50	19	81
A	A	3	1	8	40	10	81
A	B	25	17	34	93	74	98
A	C	20	14	29	85	65	94

B) Analysis

Source of variation	Num. D.f.	Initiation			Proliferation		
		Den. D.f.	F	p	Den. D.f.	F	p
Crosses	3	53	13.17	≤ 0.0001	28	3.53	0.03
AxB +AxC vs AxA[a]	(1)	(53)	30.28	≤ 0.0001	(28)	5.80	0.02
AxB vs AxC[a]	(1)	(53)	0.54	0.47	(28)	0.81	0.37
AxA vs CxA[a]	(1)	(53)	0.87	0.35	(28)	0.12	0.73

[a] a priori comparison.

Table 3 Experiment 2c with 0.2M sucrose: **A**) Average number of mature *P. sylvestris* somatic embryos g^{-1} f.m. followed by their 95 % confidence limits (Lower, Upper), per ABA concentration, per embryogenic line, and **B**) Analysis including numerator (Num.) and denominator (Den.) degrees of freedom (D.f.), F statistics (F), and their p-values (p)

A) Means

ABA (µM)	Mean	95% confidence limits	
		Lower	Upper
80	248	206	298
120	174	141	214

Cross	Line	Mean	95% confidence limits	
			Lower	Upper
A x B	MS17	57	33	98
A x B	LS8	197	157	247
A x B	MS15	198	144	270
A x B	MS6	396	358	437
A x C	LS4	441	383	507

B) Analysis

Source of variation	D. f.		F	p
	Num.	Den.		
ABA	1	98	6.26	0.014
Lines	4	98	34.98	≤ 0.0001
Cross[a]	(1)	(98)	75.04	≤ 0.0001
ABA × Lines	4	98	1.57	0.19

[a] a priori comparison.

Table 4 Experiment 2d: **A**) Average number of mature *P. sylvestris* somatic embryos g^{-1} f.m. embryogenic tissue, ratios of the means per AC level or culture age followed by their 95 % confidence limits (Lower, Upper), per AC concentration and line, per AC concentration and culture age, and per line and culture age, and **B**) Analysis including numerator (Num.) and denominator (Den.) degrees of freedom (D.f.), F statistics (F), and their p-values (p)

A) Means and ratios

Cross	Line	AC (gl^{-1}) 10	95 % confidence limits		AC (gl^{-1}) 0	95 % confidence limits		Ratio	95 % confidence limits	
			Lower	Upper		Lower	Upper		Lower	Upper
AxC	LS4	292	246	347	207	163	264	1.41	1.05	1.90
AxB	LS8	203	170	241	180	146	222	1.12	0.86	1.47
AxB	MS6	377	330	431	181	145	226	2.08	1.61	2.70

Culture age (weeks)	AC (gl^{-1}) 10	95 % confidence limits		AC (gl^{-1}) 0	95 % confidence limits		Ratio	95 % confidence limits	
		Lower	Upper		Lower	Upper		Lower	Upper
8	406	375	440	384	353	418	1.06	0.94	1.19
24	196	165	231	93	73	119	2.10	1.56	2.83

Cross	Line	Culture age (weeks) 8	95 % confidence limits		Culture age (weeks) 24	95 % confidence limits		Ratio	95 % confidence limits	
			Lower	Upper		Lower	Upper		Lower	Upper
AxC	LS4	516	474	563	117	88	156	4.40	3.27	5.93
AxB	LS8	285	254	320	128	100	164	2.23	1.70	2.92

AxB	MS6	418	379	461	163	129	208	2.56	1.97	3.31

B) Analysis

Source of variation[a]	D.f.		F	p
	Num.	Den.		
Lines	2	74	5.98	0.004
Age	1	74	229.26	≤ 0.0001
Lines × Age	2	74	6.46	0.003
Age for LS4[a]	(1)	(74)	147.51	≤ 0.0001
Age for LS8[a]	(1)	(74)	39.44	≤ 0.0001
Age for MS6[a]	(1)	(74)	64.76	≤ 0.0001
AC	1	74	25.37	≤ 0.0001
AC × Lines	2	74	5.44	0.006
AC for LS4[a]	(1)	(74)	5.43	0.02
AC for LS8[a]	(1)	(74)	0.73	0.39
AC for MS6[a]	(1)	(74)	35.53	≤ 0.0001
AC × Age	1	74	18.73	≤ 0.0001
AC for 8 weeks[a]	(1)	(74)	0.85	0.36
AC for 24 weeks[a]	(1)	(74)	25.71	≤ 0.0001
AC × Lines × Age	2	74	1.75	0.18

1 [a] a priori comparisons.

Table 5 Relative quantities of major storage protein sets in *P. sylvestris* mature somatic embryos of the white, yellow and green phenotypes in comparison with those of mature zygotic embryos, which were set to 100%.

Phenotype	Storage protein sets			
	45 kD	36 kD	22 kD	11.5 kD
White	96	78	78	74
Yellow	53	48	54	48
Green	63	34	48	47

Figure legends

Fig. 1 Clonal somatic seedling production from somatic embryos of *P. sylvestris* (A) Examples of mature somatic embryos selected for germination. Note varying numbers (3 to 7) of cotyledon primordia. (B) Germinated somatic embryo. (C) Elongated somatic seedlings at the later stages of growth compared with (B). Insert shows formation of secondary root primordia on a primary root. (D) Clonal somatic seedlings grown in a shade house for 9 months. Bar = 1mm (A), 1mm (B), 1 cm (C), 3 cm (D).

Fig. 2 Coomasie blue-stained SDS-PAGE profiles of total proteins of *P. sylvestris* zygotic embryos (z) and somatic embryos of white (sw), yellow (sy) and green (sg) phenotypes under (A) non-reducing conditions and (B) reducing conditions. Molecular mass indicators in kD are shown on the left side. Arrows and letters indicate storage proteins. Each lane received 15 µg proteins in 15 µl.