New Forests (2014) 45:1–20
DOI 10.1007/s11056-013-9388-2

Production of a new generation of seeds through the use of somatic clones in controlled crosses of black spruce (*Picea mariana*)

F. Colas · M. S. Lamhamedi

Received: 13 June 2013 / Accepted: 10 September 2013 / Published online: 26 September 2013

Abstract To assess the potential to integrate somatic clones (SC) of desired characteristics in production of high genetic quality seed, controlled crosses between different SCs of black spruce (*Picea mariana* (Mill) B.S.P.) were used to assess their suitability for the production of viable pollen, cones, seeds and seedlings. These SC produced male and female strobili at an early stage. Pollen, cones and seeds produced were characterized (mass, size, germination); their characteristics were similar to those produced by trees in natural forests or seed orchards. A maternal effect was demonstrated for the cone size and seed mass. Although seeds had excellent germination rates, the somatic biparental crosses were divided into three distinct groups with different germination curves using the Weibull function. Seeds from controlled crosses between different SC enabled the production of high morpho-physiological quality seedlings in a forest nursery. Using black spruce as a model, we showed, for the first time, that SC can be used as seed producers. These encouraging results open new perspectives on the tangible integration of somatic embryogenesis (SE) in the chain of seed, vegetative propagation (cuttings and SE) and production of plants for high productivity plantations. Controlled crosses can be made between SC with the desired characteristics (fewer large branches, fewer nodes, good growth, high wood density, performance, improved yield, etc.), vegetative propagules produced and deployed to clonal tests. After elimination of the worst performing SC, clonal tests can be converted into seed orchards that produce a new generation of seeds of high genetic quality. This will allow the rapid introduction of new materials in elite breeding programs of forest species.

Keywords Somatic clones · Flowering · Controlled crosses · Seeds · Germination · Seedlings

F. Colas (✉) · M. S. Lamhamedi
Direction de la recherche forestière, ministère des Ressources naturelles du Québec, 2700 rue Einstein, Québec, QC G1P 3W8, Canada
e-mail: fabienne.colas@mrn.gouv.qc.ca

Introduction

Black spruce [*Picea mariana* (Mill.) B.S.P.] is widely distributed in Canada and the northern United States and it grows on a wide variety of mineral and organic soils (Viereck and Johnston 1990). Black spruce (BS) is an important source of high-quality fibre for the Canadian pulp and paper industry. In boreal ecosystems, BS seedlings or trees tolerate different environmental stresses, including flooding, water stress, frost and heat stress (Grossnickle 2000; Lamhamedi and Bernier 1994).

BS is the most widely used spruce species in Québec's reforestation program. In 2012, 120 million BS seeds were shipped to 19 public and private nurseries across Québec to produce 61 million seedlings, representing 53 % of the total production in the province.

The BS genetic improvement program began in Québec in 1969 (Desponts and Numainville 2013). In addition to the production of seeds in a large network of seed orchards [close to 660 hectares (Parent 2008)], a unique model was developed in Québec for the production of BS seedlings through propagation by cuttings from the best controlled crosses (Tousignant et al. 1996). BS was used as the first model tree species in Québec to initiate work on vegetative propagation through somatic embryogenesis (SE). Early research work focussed on the development of in vitro culture protocols (Tremblay and Tremblay 1995a, b; Tremblay et al. 2005), cryogenics (Bomal and Tremblay 2000; Klimaszewska et al. 1992) and the acclimation of seedlings without the use of mist (Lamhamedi et al. 2003a). The first tests of BS somatic varieties in Québec were established in 1997. Significant enhancement has been made in the knowledge of ecophysiological processes and the use of somatic materials (El Meskaoui et al. 2000; Iraqi and Tremblay 2001; Lamhamedi et al. 2000; Vagner et al. 2005; Carrier et al. 1997a, b; Yue and Margolis 1993).

SE is a technology that allows the rapid production of genetically superior somatic clones or varieties (Park et al. 1998). Somatic clone (SC) tissues can be preserved by cryopreservation without losing their juvenility (Park et al. 1998), while providing the potential for infinite production of seedlings from the best selected SCs (Klimaszewska et al. 1992). Therefore, SE serves to reduce the time to deploy materials for reforestation by introducing superior quality clones at a much faster rate than conventional reproduction in seed orchards, because of the time needed for seed orchard trees to achieve sexual maturity (Cheliak and Rogers 1990; Högberg et al. 1998). Recent research has shown that BS SC trees become reproductive quite early (production of female strobili as early as four years after planting) without intensive tree culture (no fertilizer or flower induction treatments), that the development and receptivity of the female strobili is similar to that of female strobili produced by trees growing in the forest or in seed orchards, that they can produce seeds, and that the seeds can generate seedlings of excellent morpho-physiological quality (Colas and Lamhamedi 2009, 2010). Once initiated, female strobili production was maintained over subsequent years, showing that the trees have truly reached sexual maturity (Zimmerman 1972), even if it is early. The early production of female strobili and the development of cones was also observed with hybrid larch SCs (*Larix* × *marschlinsii* Coaz) and white spruce (*Picea glauca* (Moench) Voss) SCs at three and six years of age after planting, respectively (Colas and Lamhamedi 2009).

These quite encouraging results have provided new and very promising opportunities. The potential integration of SC into crossing schemes will serve to further enhance yields in terms of productivity and higher wood density and improve other desireable characteristics such as biotic and abiotic stress tolerance. The production of a new generation of seeds from somatic biparental crosses and their integration, for example, into the process of

propagation by cuttings and the production of seedlings for reforestation, will make important contributions to further enhancing the use of the best selected clones. This will improve the outcome of SE techniques, speeding up the progress of genetic improvement programs, and decrease the deployment time to reforestation programs.

However, our knowledge of the direct impact of SE techniques on the reproduction of forest tree species is quite limited, particularly regarding the appearance, the phenological development of male strobili, the quality of pollen produced, the level of receptivity of female strobili, and the development of cones and seeds through SCs biparental crosses. Early strobilus production has been observed for some *Picea abies* SCs (L.) Karst without specifying whether they were male or female (Helmersson et al. 2008). To our knowledge, no data have been published on the production of pollen by SCs and its viability and potential use in SC biparental crosses of any forest tree species.

To use the full potential of SE techniques for multi clonal forestry, the overall goal of this article is to determine whether SCs produce high quality pollen and if SC biparental crosses can produce seeds likely to generate seedlings of high morpho-physiological quality under the conditions found in forest tree nurseries. To do so, we used a BS SC plantation established in 1997 to conduct an in-depth assessment of tree reproduction characteristics over a long period of time. We harvested pollen from this clonal test and conducted biparental crosses. We then used the seeds to conduct germination tests and evaluate the seedlings subsequently produced.

Materials and methods

Testing sites and seedling materials

Two BS clonal tests were used. All the SCs were produced through SE using BS-specific techniques (Tremblay et al. 2005). The SCs were produced from mature seeds of non-related full-sib families selected in the BS genetic improvement program (Villeneuve 1999). Seedlings were developed from newly initiated embryogenetic tissue without going through a cryopreservation stage. For each test, all the seedlings were produced in a greenhouse at the same time.

The first clonal test was planted in August 1997 in a field of the *Centre d'expérimentation et de greffage de Duchesnay* (Centre of Experimentation and Grafting) (Long.:71°38′37″W, Lat.:46°51′05″N, Québec, Canada). The test included 16 SCs produced from six full-sib families distributed in 20 random blocks of 32 trees each. The second test was conducted in a field of the *Pépinière forestière de Saint–Modeste* (Saint–Modeste Forest Nursery) (Long.:69°23′10″W, Lat.: 47°50′10″N, Québec, Canada). The seedlings were planted on site in May 2000, and included 55 SCs produced from 20 full-sib families in 20 complete randomized blocks of 37 trees each. All the SCs used in the two tests were different. The Duchesnay test was used for pollen collection and pollination; the Saint-Modeste test was only used for pollen collection.

Seed production

In both clonal tests, male strobili were harvested manually just before pollen shedding (Beers et al. 1981). Crops ranged between 2004 and 2008, depending on the respective fructification of the SCs. In the Duchesnay test, harvests were conducted on M227, M228 and M245 SCs, and on M286, M410 and M415 SCs in the Saint-Modeste test. The six

clones were unrelated. Pollen lots were stored in the pollen bank at the Direction de la recherche forestière (DRF) of the Ministère des Ressources naturelles du Québec (MRN). Some SCs produced male strobili several times between 2004 and 2008. In these cases, the lots of each SC were stored in the bank by separate harvest year. If necessary, they were later mixed in 2009 by SC just before being used. For each SC, the viability of pollen lots was assessed using an in vitro germination test on the mixed lots, according to the procedures described by Colas and Mercier (2000).

In the Duchesnay test, six ramets of each of SCs M227, M228 and M245 were induced for female strobili production by injecting gibberellin $GA_{4/7}$ into their trunks (Pharis et al. 1987) on June 18, 2008. Each tree received a single 0.2 ml dose of a 10 % $GA_{4/7}$ solution, representing a quantity of 20 mg of phytohormone per tree. The criterion used to select SCs for female induction was the collection of pollen in previous years on the same SCs and stored in the DRF's pollen bank.

The somatic biparental crosses were conducted in Duchesnay in 2009 using the technique described by Bramlett and O'Gwynn (1981). For a single clone, each ramet (6) was pollinated with the pollen of one of the six selected SC. A pollination bag was placed over each of the six ramets of the three SCs before the beginning of female receptivity. About 0.5 ml of pollen was applied with a syringe when at least 50 % of the strobili/SC in the bag were fully receptive, i.e., when the scales of the strobili were perpendicular to the axis of the flower (Ho 1991). The date of full receptivity varied depending on the SC: May 23 for M227, May 25 for M228 and May 28 for M245. A second application of pollen was carried out two days later to maximise the chances of successful breeding. Pollination took place between May 23 and 30, 2009. Each pollination bag received a total of 1 ml of pollen. Once the strobili were closed, the pollination bags were removed. In early July, the cones were covered with a Tergal bag to protect them against insect and squirrel predation (Ho 1991).

To identify crosses, SCs used as father clones were always classified in ascending order from M227 to M415. Hence, crosses created with M227 used as mother clone were identified with numbers from 1 to 6, those created with M228 as mother clone, from 7 to 12 and those created with M245 as mother clone, from 14 to 18.

Cone harvesting, seed extraction and evaluation

Cones were harvested between August 31 and September 2, 2009. The cones were kept separated by cross and stored in a cool, well-ventilated room until seed extraction.

Shortly after harvest, but before the scales of the cones opened, the dimensions (length and width) of a representative random sample of 15 cones per cross were measured using a calliper, for each of the 18 crosses.

Seeds were extracted and sorted in January 2010, using the procedure in effect at the *Centre de semences forestières de Berthier* (Berthier Tree Seed Centre) (Brault et al. 1996). The number of seeds obtained per cross was determined with an electronic counter (Count-A-Pak®, Seedburo, Chicago, United States). For each cross, the seeds were counted twice and the mean value was used. After sorting the seeds with a gravity separator, the number of empty seeds was determined to calculate the proportion of sound seeds [number of sound seeds divided by the total number of seeds produced (sound + empty seeds)].

The 1,000-seed mass was determined using the International Seed Testing Association (ISTA 2012) procedure, except for one particular cross for which the number of seeds obtained was insufficient. The dimensions of seeds, four replicates of 100 seeds/replicate of each cross, were determined using the Winseedle® software program (Régent Instruments,

Québec, Canada) with the ellipsoidal seed shape selection. The measured variables included the straight length [straight line distance between the farthest points of each seed, mm], the straight width [maximum width measured perpendicular to the straight length, mm] and the projected area (mm^2).

Seed germination was assessed in a germinator (G30 model, Conviron, Winnipeg, Canada), in which the temperature, relative humidity, light intensity and photoperiod were controlled. Environmental conditions used were those established by ISTA (2012) for BS [21 days, 16 h of light, temperature alternation (20/30 °C), 85 % relative humidity]. For each biparental cross, four replicates of 100 seeds (the same as those used to determine the dimensions of seeds) were randomized in different germination boxes with 100 seeds/box/biparental cross. There were four different crosses per box, for a total of 400 seeds per germination box. The test was performed according to the procedures described by Wang and Ackerman (1983). All the seeds of the different crosses were sowed the same day.

To achieve a test based on random blocks, the germinator was divided into four vertical blocks and five shelves were set up to contain a total of 18 germination boxes. Therefore, one germination box of each of the blocks was located on each shelf of the germinator. In addition, for the boxes to be randomly laid out inside the germinator during the entire germination test, the positions of the shelves were changed three times a week (raised one position) and rotated.

A seed was considered to be as germinated when the cotyledons of the seedling were apparent [stage 2 described by Wang (1973)]. Seedlings were counted three times a week (Monday, Wednesday and Friday), beginning on the 7th day of germination. After each count, the seedlings were removed from the boxes to avoid counting mistakes. The germination rate as well as the germination value—a vigour index combining the rate of germination and the final proportion of germination (Czabator 1962)—were determined at the end of the test.

The final germination rate is the total number of seeds having germinated during the germination test divided by the total number of seeds sowed for germination. The accuracy of this final proportion was verified against the allowable differences table (ISTA 2012). There was no need to repeat the test. The higher the germination value, the faster the lot germinated. During germination, some albino seedlings were observed; they were counted as germinated seeds.

Production of seedlings under forest nursery conditions

The seedlings were produced at the Saint-Modeste forest tree nursery. Seeds from all biparental crosses were sowed manually on May 6, 2010 in containers (*IPL 25–310*, 25 cavities, 310 cm^3/cavity; IPL, Saint-Damien-de-Buckland, Québec, Canada). The cavities were filled with a mix of peat and vermiculite (v/v, 3/1) previously moistened and adjusted to a density of 0.1 g/cm^3 (Lamhamedi et al. 2006).

For each biparental cross, three seeds were sowed manually in each cavity of three containers to ensure obtaining one seedling per cavity, except for one lot for which two seeds were sowed per cavity due to insufficient quantities. The containers were placed in three complete randomized blocks, each including one container of each seed lot and the control lot.

Once germination was completed, about six weeks after sowing, cavities were thinned to keep only one seedling per cavity. If observed, albino seedlings were removed. No seedlings were transplanted to avoid any impact on root shape and growth. For the first year of culture, containers were placed in an unheated tunnel and outside for the second

year. Containers were surrounded by a border of containers to avoid effects on growth associated with direct radiation (e.g., rise in temperature) and significant decreases in substrate water content (Lamhamedi et al. 2006).

Fertilization and irrigation were controlled in compliance with standard BS operational production (Lamhamedi et al. 2003b). During the 2 growing seasons, mineral element inputs (N, P, K, Ca and Mg in mg/seedling) were adjusted to seedling needs using the *Plantec* software program for fertilization management in forest tree nurseries (Girard et al. 2001; Langlois and Gagnon 1993). For instance, applied quantities of nitrogen (ammonium, nitrate), phosphorous and magnesium were 19.5, 19.2, 11.6 and 20.8 mg/seedling, respectively. The fertilization program also included secondary elements and micro-elements.

To assess the growth of seedlings after the first (1 + 0) and second (2 + 0) season of culture, five seedlings were collected per container and per block by systematic random sampling. The seedlings were measured to determine their height and root collar diameter. The dry masses of shoot and roots were determined after drying seedlings for 48 h at 60 °C. The mineral element content (N, P, K, Ca and Mg) of shoot and roots was determined using a composite sample formed by five seedlings/biparental cross/block. The substrate of all the seedlings of each block was grouped together to form a composite sample to determine its mineral composition and electrical conductivity.

Mineral analyses of the seedlings and substrate were conducted by the organic and inorganic chemistry laboratory (ISO/CEI17025) at the Direction de la recherche forestière (MRN). Analysis procedures used are described by Lamhamedi et al. (2003b).

Before being planted, seedlings grown in Québec forest nurseries are subjected to a morphophysiological evaluation for 28 quality criteria (Veilleux et al. 2013). These norms are specific to each stock type and production scenario. Evaluation of (1 + 0) and (2 + 0) containerized seedlings is conducted both in the autumn and spring preceding their delivery to the planting site. For example, foliar nitrogen concentration is among the 28 quality norms that are evaluated. Large containerized BS seedlings (root plug $\geq$ 200 cm^3) must have a foliar N concentration greater than 1.8 % before being accepted for delivery to reforestation sites.

Statistical analyses

Cone, seed and plant evaluation

Analysis of variance of the variables related to the cones and seeds as well as the seedlings they produced were conducted using the MIXED procedure of the SAS/STAT® software program (SAS Institute 2009) based on the following model:

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij} \quad (1)$$

where y_{ij} = Outcome variable associated with the j-th observation of cross i, μ = General mean, α_i = Fixed effect of cross i, $i = 1,\ldots,18$, ε_{ij} = Residual error associated with the j-th observation of cross i.

When the effect of the crossing was significant, the means of the different variables were compared and grouped into non-overlapping homogenous groups determined using the Scott-Knott cluster analysis method to identify groups of non-overlapping means (Scott and Knott 1974), programmed in the R language, version 2.14.0 (Team R 2012).

Germination kinetic model

The LIFEREG procedure of SAS (SAS Institute 2009) was used to model the number of germinated seeds on the basis of the day and by crossing. The Weibull function is recommended for modeling the kinetics of seed germination (Rink et al. 1979) and comparing germination between lots (Bonner and Dell 1976). Since this procedure does not consider random factors, the sum of germinated seeds of the four blocks for each biparental cross was used as the outcome variable (100 seeds per block × 4 blocks = 400 seeds).

The Weibull function is defined by the following equation:

$$F(x) = 1 - e^{\left\{-\left[\frac{(x-a)}{b}\right]^c\right\}} \quad (2)$$

where F(x) represents the proportion of germination observed at time x. The three parameters are as follows: a = location, b = scale and c = shape. The *a* parameter is an estimate of the earliest time at which the proportion of germinated seeds is greater than zero and an indicator of the onset of germination. For the purpose of this experiment, this parameter was set at 7. The *c* parameter defines the shape of the distribution curve. The *b* parameter has a multiplicative or scaling role. When comparing two lots, a higher *b* value represents a reduction in germination. It has also been established that at the time a + b, approximately 63 % of germination is achieved (Rink et al. 1979).

The Weibull parameters of each biparental cross were estimated with the SAS LIFEREG routine (SAS Institute 2009). A cluster analysis was then used to group the germination curves using CLUSTER and TREE procedures of SAS (SAS Institute 2009). The scale (b) and shape (c) parameter estimates obtained using the Weibull function served to group together the germination curves.

Results

Male strobili production and pollen quality

The earliest SCs began producing male strobili six years after being planted and about two years after the onset of early female flower production. Eight years after planting, all the SCs in the clonal test produced male strobili, with varying production over the years. Once initiated, the production of male strobili continued on a regular basis throughout the years. The male strobili developed on the lower two-thirds of the crown, as terminal buds on branchlets.

Our observations showed that the dimensions of somatic male strobili were similar to those of male strobili produced by seed orchard trees. The time period of strobilus development and the date of pollen shedding was similar to that observed in a neighbouring seed orchard. Upon extraction, male cones produced by SCs yielded the same amount of pollen as male cones produced in a seed orchard.

The pollen used was harvested over several years due to annual variation in production. In all cases, pollen was harvested just before natural shedding: in 2006 for M286 and M410, in 2008 for M227 and M415, in 2006 and 2008 for M228, and in 2004, 2006 and 2008 for M245. On average, for the three harvest years, pollen shedding occurred between the third and fourth weeks of May for Duchesnay and between the last week in May and the first week in June for Saint-Modeste. The individual in vitro pollen germination rate of the six SCs ranged between 66 and 90 %. The great majority of pollen tubes produced were longer than at least twice the width of the pollen grain (Fig. 1).

Production of female strobili and cones

Each of the six ramets of the three selected SCs produced female strobili in sufficient quantity (>100 strobili) to conduct pollinations. Depending on the SCs, cone shape and colour were relatively homogeneous particularly for clone M245 (Fig. 1).

The cone dimensions (length and width) of somatic biparental crosses differed significantly ($p < 0.0001$) (Fig. 2a, b). Therefore, four distinct homogenous groups were identified in terms of cone length (Fig. 3a) and five distinct homogenous groups were identified in terms of cone width (Fig. 3b).

The mean length of cones produced by the three SCs was between 23.0 and 33.9 mm (Fig. 3a). The M227 SC produced the longest cones (average of 30.5 mm) and M228, the shortest (average of 26.4 mm).

The mean width of cones ranged between 10.9 and 14.8 mm (Fig. 3b). The cones produced by the M245 SC were the narrowest (Fig. 3b), and their widths were similar, regardless of the pollen used (from 11.7 to 12.7 mm, Fig. 3b).

The M228 SC produced the widest cones (mean of 14.0 mm, Figs. 2b, 3b). There were no direct relations between the number of sound seeds per cone and cone dimensions (length and width).

Seed characterization

Morphology and performance

Seeds produced by the SCs had specific tegument colors based on the maternal SC (Fig. 1).

The variables related to seed dimensions were significantly affected by the controlled cross. The Scott-Knott multiple comparison test showed that the somatic biparental crosses varied significantly ($p < 0.0001$) for the mass of 1,000 seeds and the straight seed length and width variables (Fig. 4a, b, c).

Among the three SCs used as mothers, M227 produced the heaviest seeds (1.7 g on average, Fig. 2e) and M228, the lightest seeds (1.2 g on average), for a difference of 32 %.

A significant maternal effect was highlighted for the number of sound seeds per cone ($p = 0.0041$, Fig. 2c) with SC M227 producing, on average, the largest quantity of sound seeds per cone (mean of 26.5, Fig. 2c). Similar variations were observed regarding the proportion of sound seeds produced, and the maternal effect was also significant ($p = 0.0013$); M227 produced the highest proportion of sound seeds (60.5 % on average, Fig. 2d).

Germination

Analysis of variance showed that the percentage of final germination did not vary significantly based on the somatic biparental cross ($p = 0.4782$). Mean germination of all somatic biparental crosses was 98.2 % (min. 96.5–max. 99.5 %). However, the germination value was significantly affected by the somatic biparental cross ($p < 0.0001$).

Albino seedlings were observed for some crosses during germination testing. The highest proportion was observed for the M245 inbred cross; depending on the block, the proportion was 4, 6, 10 and 14 %.

Results from analysis of seed germination using the Weibull function, combined with classification analysis, allowed for the creation of three distinct groups among biparental crosses (Fig. 5): Group 1 showed the slowest germination rate and included five of six crosses with M228 as the mother ($b = 4.69$ and $c = 2.21$, Fig. 5); Group 2 included four

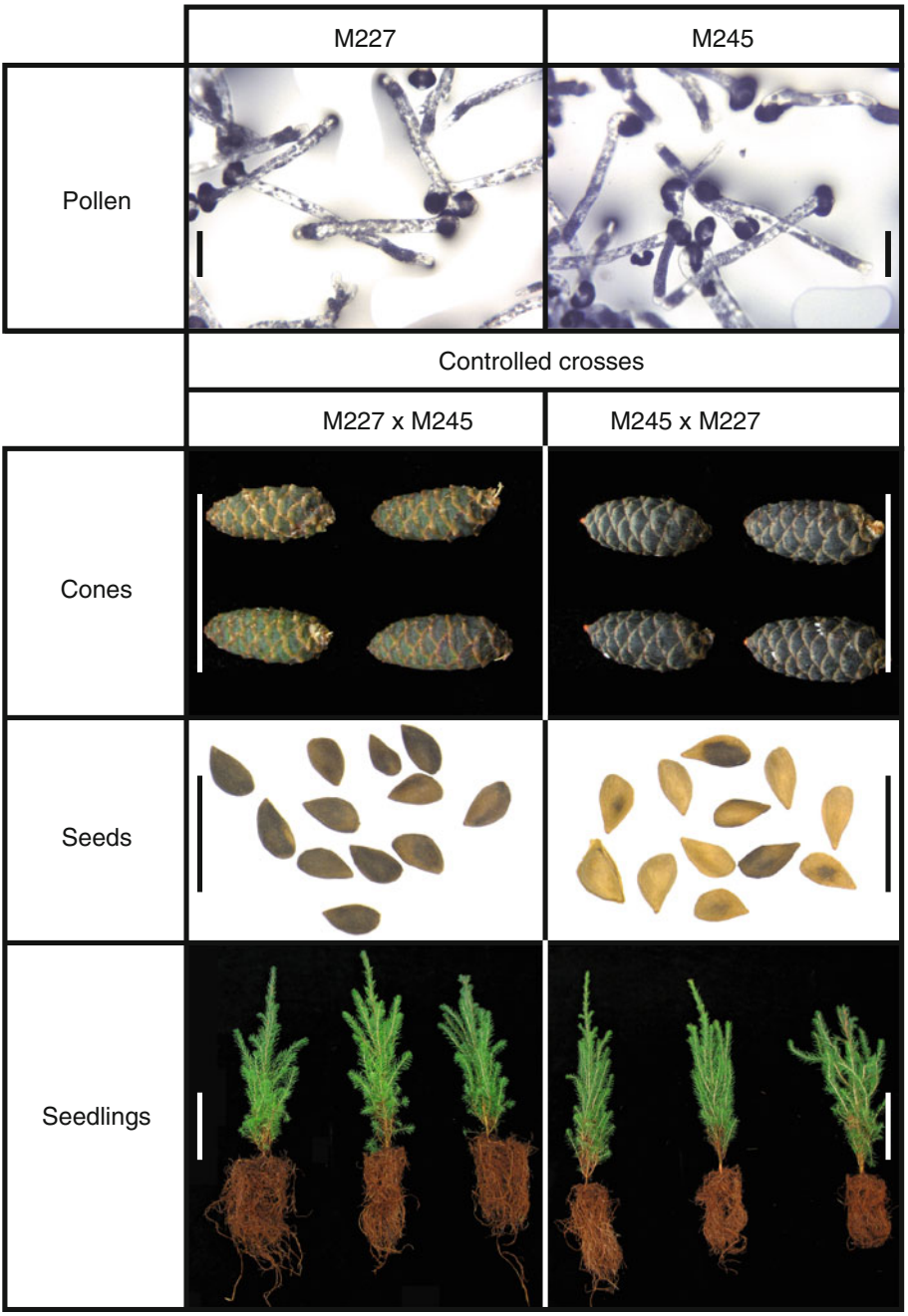


Fig. 1 Examples of different morphological development stages for two reciprocal controlled crosses with somatic clones M227 and M245. In vitro germinated pollen grains (×100 magnification, *scale bar* = 100 µm). Cones (*scale bar* = 5 cm). Seeds (*scale bar* = 5 mm). Seedlings (*scale bar* = 10 cm)

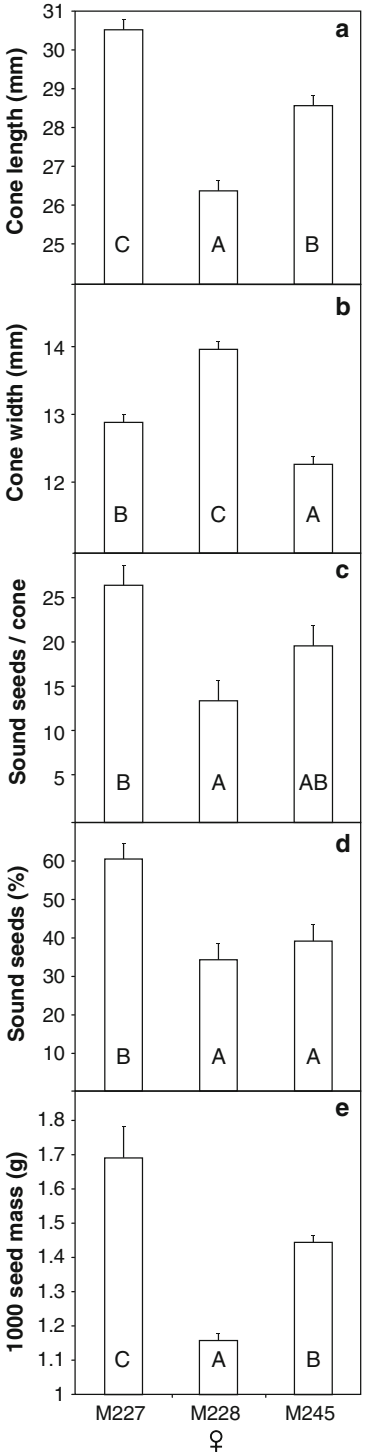


Fig. 2 Observed maternal effect regarding cone dimensions (**a** length, **b** width), the number of sound seeds per cone (**c**), the proportion of sound seeds (**d**) and the mass of 1,000 seeds (**e**)

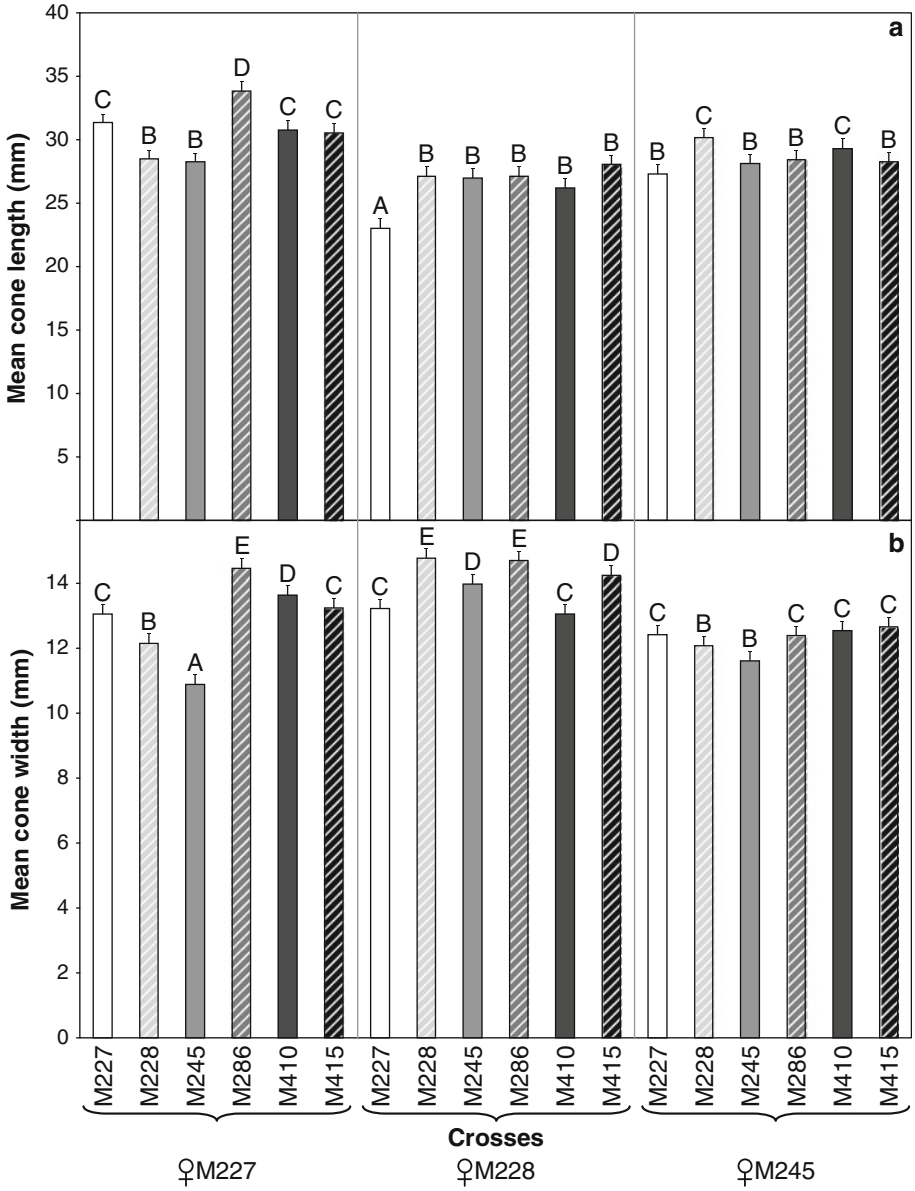
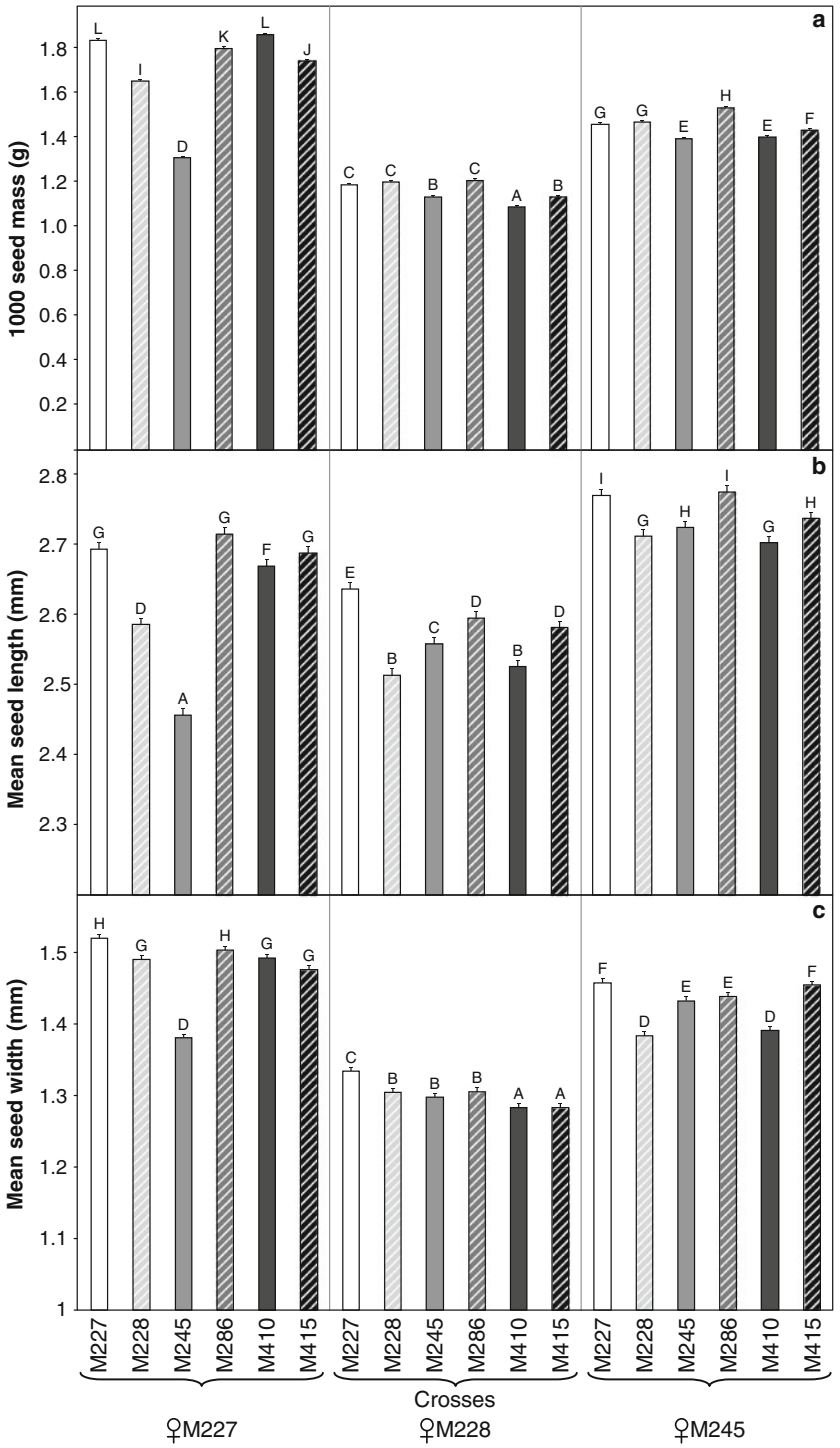


Fig. 3 Mean dimensions of cones harvested on the M227, M228 and M245 somatic clones cultivated in the Duchesnay test for the 18 crosses performed. **a** length, **b** width. *Distinct letters* represent significant differences at $\alpha = 5$ % according to Scott-Knott multiple comparison test

crosses with M245 as the mother (b = 3.42 and c = 1.60, Fig. 5); and Group 3, which included four crosses with M227 as the mother clone (b = 3.11 and c = 1.70, Fig. 5) and for which parameter b was the lowest, showed the fastest germination rate.


a
1000 seed mass (g)
b
Mean seed length (mm)
c
Mean seed width (mm)
M227
M228
M245
M286
M410
M415
Crosses
♀M227
♀M228
♀M245

◄**Fig. 4** Characterization of seeds produced by the three SCs cultivated on the Duchesnay site. **a** mass of 1,000 seeds (g); **b** mean length (mm); and **c** mean width (mm). *Distinct letters* represent significant differences at 5 % according to Scott-Knott multiple comparison test

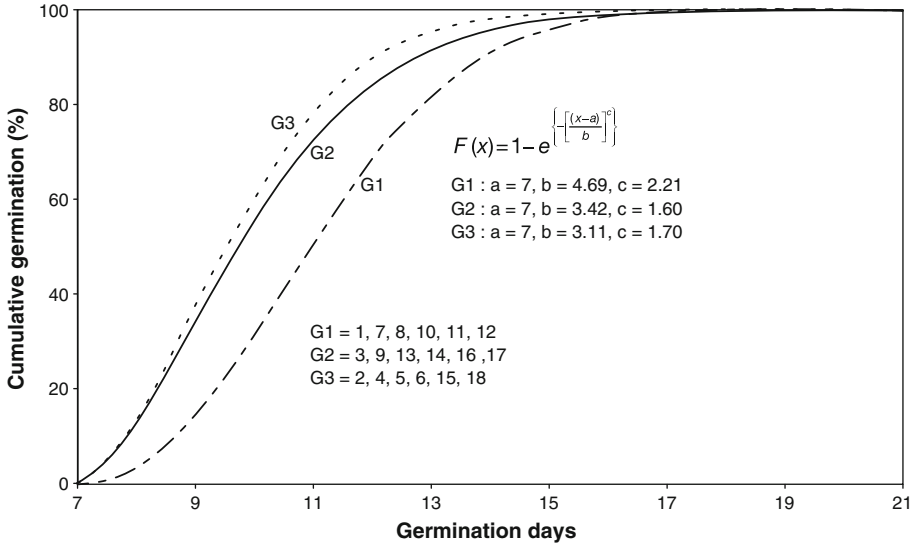


Fig. 5 Cumulative germination of the three groups of somatic biparental crosses obtained with the Weibull function

Seedling growth and mineral nutrition

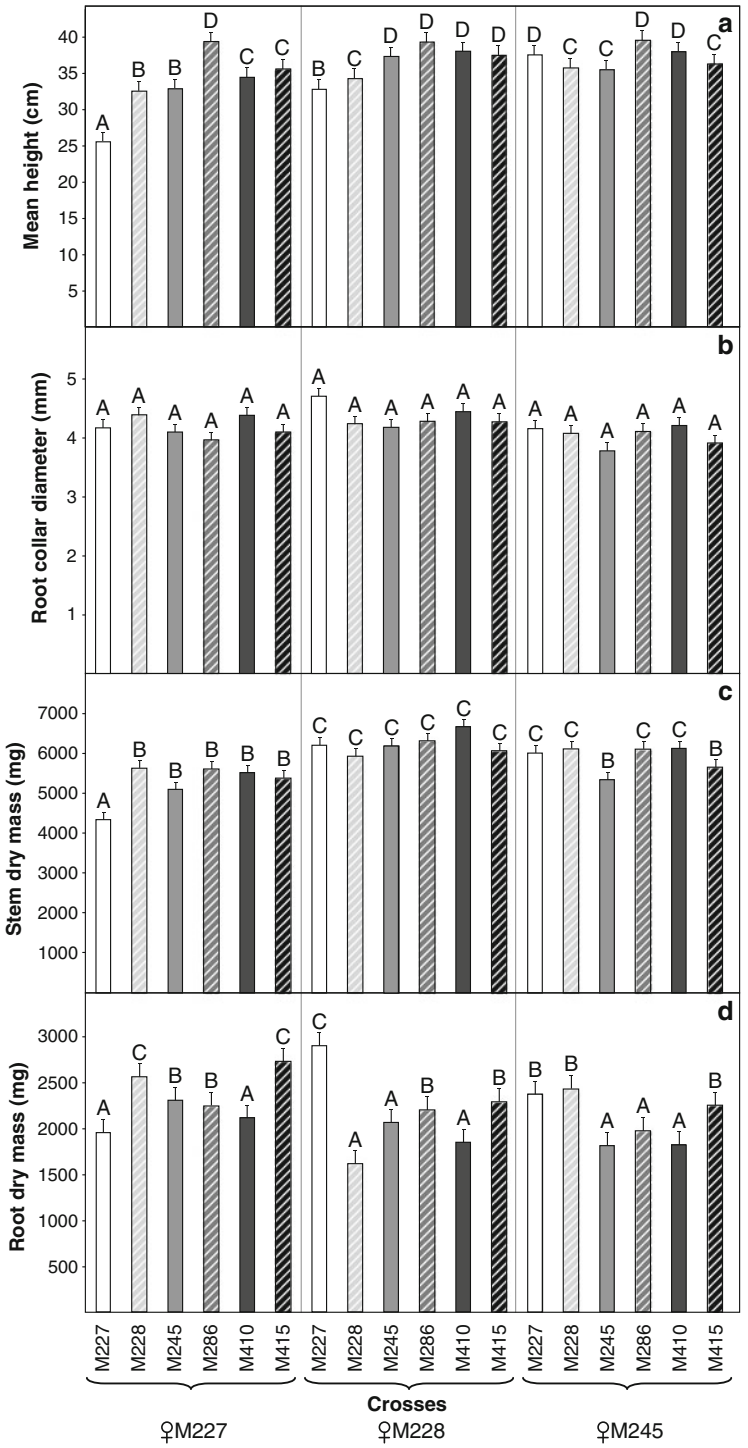
The different growth variables were significantly affected by the somatic biparental cross: height ($p = 0.0017$), root collar diameter ($p = 0.0003$), root dry mass ($p < 0.0001$), shoot dry mass ($p < 0.0001$), and total dry mass of seedlings ($p = 0.0005$). Therefore, after two years of growth in a forest tree nursery, seedlings achieved variable sizes depending on the parents (Fig. 1). Mean comparisons, based on the Scott-Knott test, showed the presence of different groups of distinct somatic biparental crosses depending on the growth variable (Fig. 6). Based on the variable, three to four distinct homogenous groups were created (Fig. 6a, c, d). It should be noted that the analysis of variance showed a significant difference for root collar diameter, but no groups were created with the Scott Knott test (Fig. 6b).

The mineral element concentrations in the plant shoots for all the controlled crosses were 3.19 % for N, 0.54 % for P and 1.41 % for K. Concentrations for the same elements in the roots were 3.53, 0.79 and 1.90 %, respectively.

Generally, the majority of plants produced from different controlled crosses meet the morphophysiological standards of quality used in Québec.

Discussion

Using black spruce as a model, we showed for the first time that somatic clones (SC) can be used to produce seed. Indeed, SCs can produce male strobili and good quality pollen suitable for pollinating female strobili and producing high-quality seeds and seedlings. The arrangement and location of male strobili was in the lower two-thirds of the tree crown, at


a
Mean height (cm)
b
Root collar diameter (mm)
c
Stem dry mass (mg)
d
Root dry mass (mg)
M227
M228
M245
M286
M410
M415
Crosses
♀M227
♀M228
♀M245

◄**Fig. 6** Height (**a**), root collar diameter (**b**), dry mass of shoot (**c**) and root (**d**) of seedlings produced by the 18 black spruce somatic biparental crosses after two years of culture in a nursery. *Distinct letters* represent the significant differences at $\alpha = 5$ % and the identification of homogenous groups according to Scott–Knott multiple comparison test

the end of branchlets, which is similar to that observed on trees of this species in both natural forests and seed orchards (Ho 1991; Powell 2007).

These results reinforce the previously demonstrated observation that SCs can produce fertile female strobili for producing seeds and seedlings of high morpho-physiological quality (Colas and Lamhamedi 2010). Owing to these breakthroughs, the faster integration of elite SCs into somatic biparental cross plans can now be considered to generate seeds to be used in the production of somatic stock plants for the propagation of forest tree seedlings by cuttings, or somatic embryogenesis. Likewise, when genetic testing allows identifying the best individuals and eliminating lower-yield individuals, it will become appropriate to convert clonal tests into seed orchards. Seeds produced with elite SC crosses will contribute to rapid enhanced genetic gains. This approach will serve to promote the use of SC tests and rapidly integrate progress made in the selection of elite clonal material.

The number of SCs to preserve in a future seed orchard must be sufficient to maintain high levels of genetic diversity. Before converting clonal tests into seed orchards, the flowering potential of elite clones will have to be evaluated in the long term. When selecting SCs, potential variations in tree fertility likely to affect the production of seedlots with balanced genetic composition have to be considered. Indeed, the contribution of different clones in a seed orchard is well-known to vary from year to year, and some clones can be dominant in seedlots or, conversely, seldom present and can, therefore, affect the optimal expression of genetic gain anticipated by breeders (Bilir et al. 2003).

In the studied clonal tests, male strobili production occurred two years after the first production of female strobili. For the studied SCs, once initiated, male strobili production continued over time, showing that the trees had reached sexual maturity (Zimmerman 1972). Like the female cones, male cones appeared early in the development of the trees. The first male strobili production occurred seven years after plantation, while it is around ten years in natural forests (Caron and Powell 1989).

The early maturation of forest trees induced by in vitro culture has been highlighted by several authors (e.g., Greenwood 1995). But, the causes of early male and female strobili production, among somatic forest tree clones remain undocumented. However, in soybean, genes controlling flowering have been recently identified (Xia et al. 2012).

In germination tests conducted on the pollen lots used in this experiment, the majority of pollen tubes produced were longer than at least twice the pollen grain width. This is actually the criterion used to identify pollen as germinated (Colas and Mercier 2000), and it reflects the excellent quality of pollen lots produced by SCs.

Regardless of the SC, female strobili developed in the upper one-third of tree crowns. The same was observed for this tree species in natural forests and plantations (Ho 1991; Caron and Powell 1992; Powell 2007). The dates of female flower receptivity and maturity for cone harvest were also similar to those observed for trees of natural forest and seed orchard (Caron and Powell 1992).

The length of cones measured for the three SCs as well as the mass of 1,000 seeds were comparable to those presented by Caron and Powell (1989) for young BS plantation trees aged from 8 to 16 years.

The M227 SC produced the highest number and proportion of sound seeds per cone and the largest mass of 1,000 seeds, regardless of the pollen used. M227 also produced long

cones. This is consistent with the fact that the largest cones contain the heaviest seeds (Skeates and Haavisto 1995). The M228 SC was less fertile and produced lighter seeds. Like natural origin trees, BS SCs showed a strong maternal effect on the fertility of crosses (Castro et al. 2008) and seed mass (Harper et al. 1970). No paternal effect was demonstrated since, for the same pollen used, results obtained with seeds are highly variable depending on the SC used. It should be noted that a paternal effect has been shown with *Castanea* controlled crosses (Worthen and Woeste 2007).

In a previous study (Colas and Lamhamedi 2010), the M227, M228 and M245 SCs were pollinated with pollen generated from a 1st generation BS seed orchard. The masses of 1,000 seeds were quite close to those obtained in this study. This finding shows that the differences observed were due to a maternal effect rather than environmental conditions prevailing during pollination and seed development (Thapliyal et al. 2008).

Although seeds had excellent germination rates, the somatic biparental crosses were divided into three distinct groups, using the Weibull function (Bonner and Dell 1976), with different germination curves. Each group generally included crosses with the same mother clone, showing that genetic selection based on germination is possible. A maternal effect related to the proportion of sound seeds and the mass of 1,000 seeds was also demonstrated. This finding suggests that the selection of SCs can be targeted based on seed characteristics. As a matter of fact, heavier black spruce seeds produce more vigourous seedlings (Skeates and Haavisto 1995) and there is a positive link between seed and seedling dimensions of white spruce (Burgar 1964).

Inbred crosses were carried out as part of a 6 × 6 di allele crossing plan (Zobel and Talbert 1984). The proportion of sound seeds obtained varied based on the SC. Seed dimensions and mass were comparable to those of seeds obtained through unrelated crosses using the same mother clone. And, as expected, a variable proportion of albino seedlings was observed during germination (Nanson 2004). The phenomenon of incompatibility in spruce trees is expressed by seed non-viability to avoid inbreeding depression (Hagman 1975). However, the inbred seedlings had a lower total dry mass than the seedlings obtained from other crosses involving the same mother clone.

Seedlings from non-inbred crosses developed in the same manner as seedlings of this tree species grown through standard nursery production. This finding showed that seed produced from somatic biparental crosses develop into seedlings with a morpho-physiological quality comparable to those grown from conventional seed orchard seed and that the clonal tests, after selection of the best individuals, can be converted into seed orchards. In addition, with the advances in molecular genetics related to marker-assisted selection for desired characteristics like wood density (Beaulieu et al. 2011) and growth (MacKay et al. 2011), elite individuals, selected for particular criteria, could be used in specific crossings plans. This will produce new material of high genetic quality to complement the selections already made from conventional breeding programs.

In the seedlings, mineral concentrations were similar to those observed during standard operational production. For example, BS seedlings of different controlled crosses showed higher foliar N concentration than 1.8 %. This finding shows that, although generated by seeds produced by SCs, these seedlings are nutritionally the same as seedlings generated from orchard seed. This finding confirms that SCs can actually be considered as seed producers.

The wide variability of somatic biparental crosses observed in terms of fertility showed that this factor should be considered when selecting SCs for the conversion of clonal tests into seed orchards. If this criterion is not considered, there is a risk of creating orchards where some clones, despite their high-performance for traits such as growth or low branching, have low fertility for the production of high genetic quality seeds.

Conclusion

The ability of SCs to produce pollen and seeds is valuable to vegetative propagation through SE. Seedlings produced through SE and selected for their exceptional performance would no longer be used solely for reforestation as part of multi clonal plantations, but also as high genetic quality pollen and seed producers. The operational integration of SE into the management of seed orchards for the production of seeds with an exceptional genetic gain will serve to establish sound scientific foundations for highly productive multi clonal forestry, while respecting the maintenance of genetic diversity.

Acknowledgments The authors wish to thank Mr. Carol Parent at the Direction de la recherche forestière (DRF) of the Ministère des Ressources naturelles (MRN) for the pollinations, the extraction and qualification of seeds. Many thanks go to Ms. Louise L'Heureux, Mr. Patrick Lemay and Mr. Michel Houle at the DRF for the seedling treatment, as well as Ms. Linda Veilleux, Ms. Maripierre Jalbert and Ms. Brigitte Boudreault at the DRF for the photomontage. We also thank Dr. Hank Margolis, the associate editor and the two anonymous reviewers for their valuable comments and suggestions. The authors thank the Saint-Modeste nursery (MRN) for the organization of work (Mr. Michel Rioux), pollen harvesting (Mr. Paul-Yvan Martin), and seedling production (Ms. Corine Rioux). Lastly, the authors thank Ms. Geneviève Picher and Ms. Jessica Bach from the DRF biometrics team for the statistical analyses and the DRF organic and inorganic chemistry laboratory staff for the mineral analyses of the substrates and seedlings produced during this experiment. This research work was completed as part of the 1120549-112310084 project funded by the DRF.

References

Beaulieu J, Doerksen T, Boyle B, Clément S, Deslauriers M, Beauseigle S, Blais S, Poulin P-L, Lenz P, Caron S, Rigault P, Bicho P, Bousquet J, MacKay J (2011) Association genetics of wood physical traits in the conifer white spruce and relationships with gene expression. Genetics 188(1):197–214. doi:10.1534/genetics.110.125781

Beers WL, Bivens J, Mocha JE (1981) Pollen collection. In: Franklin EC (ed) Pollen Management Handbook, vol Agriculture Handbook 587. vol 30–32. USDA, Forest Service, Washington, DC (USA), pp 30–36

Bilir N, Kang K-S, Lindgren D (2003) Fertility variation and effective number in the seed production areas of *Pinus radiata* and *Pinus pinaster*. Silvae Genetica 52(2):75–77

Bomal C, Tremblay F-M (2000) Dried cryopreserved somatic embryos of two *Picea* species provide suitable material for direct plantlet regeneration and germplasm storage. Ann Bot 86:177–182

Bonner FT, Dell TR (1976) The Weibull function: a new method of comparing seed vigor. J Seed Technol 1(1):96–103

Bramlett DL, O'Gwynn CH (1981) Controlled pollination. In: Franklin EC (ed) Pollen management handbook. vol 10–14. USDA, Forest Service, Washington, DC, pp 44–51

Brault N, Mercier S, Bettez M (1996) Traitement des graines d'arbres forestiers: 2^{ième} partie de 2. L'Aubelle 113(37):1–12

Burgar RJ (1964) The effect of seed size on germination, survival and initial growth in white spruce. For Chron 40(1):93–97

Caron G-É, Powell GR (1989) Cone size and seed yield in young *Picea mariana* trees. Can J For Res 19:351–358

Caron G-É, Powell GR (1992) Patterns of cone distribution in crowns of young *Picea mariana*. I. Effect of tree age on seed cones. Can J For Res 22:46–55

Carrier DJ, Bock CA, Cunningham JE, Cyr DR, Dunstan DI (1997a) (+)-ABA content and lipid deposition in Interior spruce somatic embryos. In Vitro Cell Dev Biol Plant 33(3):236–239

Carrier DJ, Cunningham JE, Taylor DC, Dunstan DI (1997b) Sucrose requirements and lipid utilization during germination of interior spruce (*Picea glauca engelmannii* complex) somatic embryos. Plant Cell Rep 16(8):550–554. doi:10.1007/s002990050277

Castro J, Reich PB, Sanchez-Miranda A, Guerrero JD (2008) Evidence that the negative relationship between seed mass and relative growth rate is not physiological but linked to species identity: a within-family analysis of Scots pine. Tree Physiol 28:1077–1082

Cheliak WM, Rogers DL (1990) Integrating biotechnology into tree improvement programs. Can J For Res 20(4):452–463. doi:10.1139/x90-062

Colas F, Lamhamedi MS (2009) Integration of somatic clones in seed orchard management and the production of a new generation of seeds with a high genetic value. Tree Seed Work Group News Bulletin 50:27–31

Colas F, Lamhamedi MS (2010) Floraison précoce et production de graines par des clones somatiques d'épinette noire (*Picea mariana*): intégration potentielle dans le programme d'amélioration génétique et l'aménagement des vergers à graines. Can J For Res 40(7):1421–1433

Colas F, Mercier S (2000) Évaluation et maintien de la viabilité des pollens utilisés dans le programme d'amélioration des arbres. Gouvernement du Québec, Ministère des Ressources naturelles, Forêt Québec, Direction de la Recherche Forestière, Sainte-Foy. Mémoire de recherche forestière 135. 78 p

Czabator FJ (1962) Germination value: an index combining speed and completeness of pine seed germination. For Sci 8(4):386–396

Desponts M, Numainville G (2013) L'amélioration génétique de l'épinette noire au Québec. Bilan et perspectives. Gouvernement du Québec, ministère des Ressources naturelles, Direction de la recherche forestière, Québec. Mémoire de recherche forestière 169. 42 p

El Meskaoui A, Desjardins Y, Tremblay FM (2000) Kinetics of ethylene biosynthesis and its effects during maturation of white spruce somatic embryos. Physiol Plantarum 109:333–342

Girard D, Gagnon J, Langlois C-G (2001) Plantec: un logiciel pour gérer la fertilisation des plants dans les pépinières forestières. Gouvernement du Québec, Ministère des Ressources naturelles, Direction de la recherche forestière. Note de recherche forestière 111. 8 p

Greenwood MS (1995) Juvenility and maturation in conifers: current concepts. Tree Physiol 15(7–8):433–438. doi:10.1093/treephys/15.7-8.433

Grossnickle S (2000) Ecophysiology of northern spruce species. The performance of planted seedlings. National Research Council of Canada, Canada, 407 p

Hagman M (1975) Incompatibility in forest trees. Proc Royal Soc Lond Ser B 188:313–326

Harper JL, Lovell PH, Moore KG (1970) The shapes and sizes of seeds. Ann Rev Ecol Syst 1(1):327–356. doi:10.1146/annurev.es.01.110170.001551

Helmersson A, Jansson G, Bozhkov PV, Von Arnold S (2008) Genetic variation in microsatellite stability of somatic embryo plants of *Picea abies*: a case study using six unrelated full-sib families. Scand J For Res 23(1):2–11

Ho RH (1991) A guide to pollen—and seed-cone morphology of black spruce, white spruce, jack pine and eastern white pine for controlled pollination. Forest Research Report 125. Ministry of Natural Resources, Ontario Forest Research Institute, Sault Sainte-Marie, Ont. 31 p

Högberg K-A, Ekberg J, Norell L, von Arnold S (1998) Intergration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Can J For Res 28(10):1536–1545

Iraqi D, Tremblay FM (2001) The role of sucrose during maturation of black spruce [*Picea mariana* (Mill.) BSP] and white spruce [*Picea glauca* (Moench) Voss] somatic embryos. Physiol Plantarum 111(3):381–388

ISTA (2012) International Rules for Seed testing. Bassersdorf (Switzerland)

Klimaszewska K, Ward C, Cheliak WM (1992) Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix* x *eurolepis*) and black spruce (*Picea mariana*). J Exp Botany 43(246):73–79

Lamhamedi MS, Bernier PY (1994) Ecophysiology and field performance of black spruce (*Picea mariana*): a review. Ann Sci For 51:529–551

Lamhamedi MS, Chamberland H, Bernier PY, Tremblay FM (2000) Clonal variation in morphology, growth, physiology, anatomy and ultrastructure of container-grown white spruce somatic plants. Tree Physiol 20:869–880

Lamhamedi MS, Chamberland H, Tremblay FM (2003a) Epidermal transpiration, ultrastructural characteristics and net photosynthesis of white spruce somatic seedlings in response to in vitro acclimatization. Physiol Plantarum 118:554–561

Lamhamedi MS, Margolis HA, Renaud M, Veilleux L, Auger I (2003b) Effets de différentes régies d'irrigation sur la croissance, la nutrition minérale et le lessivage des éléments nutritifs des semis d'épinette noire (1 + 0) produits en récipients à parois ajourées en pépinière forestière. Can J For Res 33:279–291

Lamhamedi MS, Labbé L, Margolis HA, Stowe DC, Blais L, Renaud M (2006) Spatial variability of substrate water content and growth of white spruce seedlings. Soil Sci Soc Am J 70:108–120

Langlois C-G, Gagnon J (1993) A global approach to mineral nutrition based on the growth needs of seedlings produced in forest tree nurseries. In: Barrow NJ (ed) Plant nutrition—from genetic engineering to field practice, 1993. Kluwer Academic Publishers, Dordrecht, pp 303–306

MacKay J, Boyle B, El Kayal W, Namroud MC, Doerksen T, Cooke J, Isabel N, Beaulieu J, Rigault P, Bicho P, Bousquet J (2011) Gene mapping in white spruce (P. glauca): QTL and association studies integrating population and expression data. BMC Proceedings 5 (Suppl 7):I6

Nanson A (2004) Génétique et amélioration des arbres forestiers. Les Presses Agronomiques de Gembloux, Gembloux (Belgique). 712 p

Parent B (2008) Aménagement forestier—Production de plants (chapitre 5). In: Ministère des Ressources naturelles et de la Faune. Ressources et industries forestières—Portrait statistique—Édition 2008. Gouvernement du Québec, Québec, pp 05-05-01 à 05-06-34

Park YS, Barrett JD, Bonga JM (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34(3):231–239

Pharis RP, Webber JE, Ross SD (1987) The promotion of flowering in forest trees by gibberellin A4/7 and cultural treatments: a review of the possible mechanisms. For Ecol Manag 19(1–4):65–84. doi:10.1016/0378-1127(87)90012-0

Powell GR (2007) Lives of conifers. A comparative account of the coniferous trees indigenous to north-eastern North America. Fitzhenry and Whiteside Ltd, Markham. 276 p

Rink G, Dell TR, Switzer G, Bonner FT (1979) Use of the Weibull function to quantify sweetgum germination data. Silvae Genetica 28(1):9–12

SAS Institute Inc. (2009) SAS/STAT® 9.2 User's guide, Second Edition. Cary, NC (USA)

Scott RJ, Knott M (1974) A cluster analysis method for grouping means in the analysis of variance. Biometrics 30:507–512

Skeates DA, Haavisto VF (1995) Heavier black spruce seeds produce more vigorous seedlings. Technical note edn. Natural resources Canada, Canadian Forest Service-Ontario. Technical Note 31. 4 p

Team R (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna (Austria)

Thapliyal M, Singh O, Sah B, Bahar N (2008) Seed source variation and conservation of *Pinus wallichiana* in India. Ann For Res 51:81–88

Tousignant D, Périnet P, Rioux M (1996) Black spruce cutting propagation at the Pépinière de Saint-Modeste. edn. Gouvernement du Québec, Ministère des Ressources naturelles. 33 p

Tremblay L, Tremblay FM (1995a) III. 14 Somatic embryogenesis in black spruce [*Picea mariana* (Mill.) B.S.P.] and red spruce (*P. rubens* Sarg.). In: Bajaj YPS (ed) Somatic embryogenesis and synthetic seed I—biotechnology in agriculture and forestry, vol 30. Springer, Berlin, Heidelberg, pp 431–445

Tremblay L, Tremblay FM (1995b) Maturation of black spruce somatic embryos: sucrose hydrolysis and resulting osmotic pressure of the medium. Plant Cell Tissue Organ Cult 42(1):39–46

Tremblay F-M, Iraqi D, El Meskaoui A (2005) Protocol of somatic embryogenesis: Black spruce (*Picea mariana* (Mill.) B.S.P.). In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants, vol 77. Forestry Sciences. Springer, Dordrecht, pp 59–68

Vagner M, Fischerova L, Spackova J, Vondrakova Z (2005) Somatic embryogenesis in Norway spruce. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants, vol 77. Forestry Sciences. Springer, Dordrecht. pp 141-155

Veilleux P, Allard J-Y, Bart F, Boyer Groulx D, Gingras B-M, Labrecque D, Marchand P, Murray A (2013) Guide terrain. Inventaire de qualification des plants résineux cultivés en récipients. Document de travail, livraison 2013. Gouvernement du Québec, ministère des Ressources naturelles et de la Faune, Direction générale des pépinières et des stations piscicoles. 141 p

Viereck LA, Johnston WF (1990) Black spruce. Picea mariana (Mill.) B.S.P. In: Burns RM, Honkala BH (eds) Silvics of North America, Vol 1: conifers. Agriculture handbook 654. U.S Department of Agriculture, Forest Service, Washington DC (USA). 22 p

Villeneuve M (1999) Les programmes d'amélioration génétique: bilan des réalisations. L'épinette noire. In: L'amélioration génétique en foresterie: où en sommes-nous?, Rivière-du-Loup, Québec, 28–30 sept. 1999, pp 45–52

Wang BSP (1973) Laboratory germination criteria for red pine (*Pinus resinosa* Ait.) seed. Proc Assoc Off Seed Anal 63:94–101

Wang BSP, Ackerman F (1983) A new germination box for tree seed testing. Environment Canada, Canadian Forestry Service, Petawawa National Forestry Institute. Information Report PI-X-27F. 15 p

Worthen L, Woeste K (2007) Male genotype influences seed set and seed size in controlled crosses of American Chestnut (*Castanea dentata* [Marsh] Borhk). In: 29th Southern Forest Tree Improvement Conference. Tree improvement in North America: Past, Present, and Future, Galveston (Texas, USA), June 19–22, 2007. pp 176–177

Xia Z, Zhai H, Liu B, Kong F, Yuan X, Wu H, Cober E, Harada K (2012) Molecular identification of genes controlling flowering time, maturity, and photoperiod response in soybean. Pl Syst Evol 298(7):1217–1227. doi:10.1007/s00606-012-0628-2

Yue D, Margolis HA (1993) Photosynthesis and dark respiration of black spruce cuttings in response to light and temperature. Can J For Res 23:1150–1155

Zimmerman RH (1972) Juvenility and flowering in woody plants: a review. HortScience 7(5):447–455

Zobel B, Talbert J (1984) Applied forest tree improvement. Wiley, New York. 505 p