A novel approach for the operational production of hybrid larch seeds under northern climatic conditions

by Fabienne Colas[1,2], Martin Perron[1], Denise Tousignant[1], Carol Parent[1], Monique Pelletier[1] and Patrick Lemay[1]

ABSTRACT

Hybrid larch ([*Larix* × *marschlinsii* Coaz], HL) is sought after by the forest industry because of its growth characteristics, excellent wood quality and disease resistance. However, the difficulty in obtaining HL seeds limits the production of seedlings for reforestation. Both European larch (*Larix decidua* Mill.) and Japanese larch (*Larix kaempferi* [Lamb.] Carrière) produce few seeds and the time lag in their flowering phenology complicates their natural pollination. We have developed a novel type of sheltered seed orchard, dedicated to the production of HL seeds from grafts grown in pots. Inverting the arches of a conventional tunnel provides a sturdy structure which is easy to maintain. Because of its height, the tunnel can accommodate the rapid growth of the trees, which reduces the need for pruning. The tunnels are covered in the winter and spring. In the winter, the covers prevent snow accumulation. In the spring, they create a "greenhouse effect" inside the tunnels, accelerating flower development, preventing pollen contamination from trees outside the tunnels and protecting the flowers from late spring frosts. Over the past five years, flowering has been regular and abundant. Bagging the crowns for pollen harvest is cost-effective and yields large quantities of high quality pollen. Pollination operations are conducted using an electrostatic pistol. Because of the large number of available clones, large quantities of seeds with a high genetic variability can be produced at a competitive cost. In 2006, this seed orchard concept was implemented operationally at the Berthier forest nursery (Québec, Canada).

Key words: Japanese larch (*Larix kaempferi* [Lamb.] Carrière), European larch (*Larix decidua* Mill.), seed production, electrostatic pollination, indoor seed orchard

RÉSUMÉ

Le mélèze hybride ([*Larix* × *marschlinsii* Coaz], MEH) est recherché par l'industrie forestière pour sa croissance, son bois de qualité et sa résistance aux maladies. Toutefois, la difficulté d'obtenir des graines de MEH limite la production de plants pour le reboisement. En effet, les mélèzes d'Europe (*Larix decidua* Mill.) et du Japon (*Larix kaempferi* [Lamb.] Carrière) produisent peu de graines, et le décalage phénologique dans leur floraison complique la pollinisation naturelle. Nous avons développé un nouveau type de verger sous abri, dédié à la production de graines de MEH à partir de greffes cultivées en pots. L'inversion des arches sur un tunnel conventionnel fournit une structure peu onéreuse, facile d'entretien et accommodant bien la croissance des arbres, limitant les besoins d'étêtage. Des toiles recouvrent le tunnel en hiver et au printemps. L'hiver, elles évitent l'accumulation de neige. Au printemps, elles créent un « effet de serre », accélérant le développement phénologique des fleurs et évitant la contamination pollinique provenant des arbres extérieurs, tout en protégeant les fleurs contre les gels tardifs. Depuis cinq ans, la floraison des arbres est régulière et abondante. La récolte de pollen, par ensachage des cimes, permet d'obtenir de grandes quantités de pollen de qualité. Les pollinisations sont réalisées avec un pistolet électrostatique. Grâce au nombre élevé de clones disponibles, nous pouvons produire de grandes quantités de graines, avec un coût de revient relativement faible, tout en maintenant une diversité génétique élevée. Ce concept de verger à graines a été transféré à l'échelle opérationnelle en 2006 à la pépinière forestière de Berthier (Québec, Canada).

Mots-clés : Mélèze du Japon (*Larix kaempferi* [Lamb.] Carrière), mélèze d'Europe (*Larix decidua* Mill.), production de semences, pollinisation électrostatique, verger à graines sous abri

[1]Ministère des Ressources naturelles et de la Faune, Direction de la recherche forestière, 2700, rue Einstein, Québec (Québec) G1P 3W8.
[2]Corresponding author. E-mail: fabienne.colas@mrnf.gouv.qc.ca

Introduction

The Québec government has led a genetic improvement program for larch since the 1970s. The program first focused on the introduction of exotic species such as European larch (EL) (*Larix decidua* Mill.) and Japanese larch (JL) (*Larix kaempferi* [Lamb.] Carrière) and the testing of their capacity to adapt to certain ecological regions in the province of Québec. In the mid-1980s, the Québec program began to conduct crossings between the best EL and JL individuals, with the goal of developing hybrid larch plants (HL [JL × EL and EL × JL]; *Larix × marschlinsii* Coaz) adapted to certain Québec ecological domains and the demands of the Québec forest industry. HL has several desirable qualities, such as rapid growth, superior wood quality, and disease and pest resistance (particularly to needle casts, larch canker and larch casebearer), for which it is sought after by the forest industry (Tousignant and Stipanicic 2000a). The HL varieties that are presently available in Québec should be able to produce average annual growth rates between 2.3 $m^3 \cdot ha^{-1}$ and 8.5 $m^3 \cdot ha^{-1}$ at 30 years of age (Bolghari and Bertrand 1984), depending on site quality. The high yields obtained in both operational and experimental plantations (Stipanicic 1999), as well as the quality of wood produced (Chui and MacKinnon-Peters 1995), is stimulating a demand for HL seedlings, 500 000 of which will be available for planting in 2007.

Seed production of larches, like other conifers, varies greatly from year to year (Rudolf 1974; Tousignant and Stipanicic 2000b, c). For both EL and JL, the interval between good seed years varies from three to 10 years (Rudolf 1974). In addition, larches are considered to be poor seed producers. This can be explained by several factors: poor pollen production (Owens and Molder 1979), insufficient female flowers and poor pollination (Kosinski 1986), or embryo degeneration (Slobodnik and Guttenberger 2000, 2005). Furthermore, larches flower very early in the spring. This makes the flowers, and the pollination process itself, vulnerable to adverse meteorological conditions (rain, snowfall, late spring frost), which may negatively affect seed production (Lee 2003).

EL and JL hybridize naturally and different seed orchard concepts have been developed to help the natural production of HL seeds (Häcker and Bergmann 1991, Tröber and Haasemann 2000, Kuromaru *et al.* 2004). However, the proportion of hybrid seeds is very variable. Seed lots produced in open-pollinated seed orchards tend to be a mix of hybrid and pure species seeds, depending on the parent tree from which they are harvested (Acheré *et al.* 2004, Pâques *et al.* 2006). Therefore, in order to guarantee high proportions of HL seeds in seed lots, it is necessary to resort to artificial pollination.

In Québec, HL seeds produced by controlled crossings are used to propagate plants from rooted cuttings (Tousignant and Rioux 2002). This enables producers to make the best use of the limited quantity of high quality HL seed. Until 2003, these seeds came from controlled field crosses of selected trees, using pollination bags. It was difficult to obtain a regular seed supply, given that irregular flowering and unpredictable climatic conditions often resulted in poor seed production.

Traditionally, controlled crossings are carried out in pollination bags. The female flowers are isolated and pollinated artificially (Bramlett and O'Gwynn 1981). A prototype of a pollination cage for larches, attached to a tractor, was developed in France (Philippe and Baldet 1997, Baldet 2006) with the goal of carrying out large-scale pollinations of plantation-grown trees. According to the most recently published results, the proportion of hybrid seeds produced is 93%, which is extremely high for trees cultivated outside, where flower and seed development depend on environmental conditions (Philippe *et al.* 2001).

Indoor seed orchards, in which the potted parent trees are kept under shelters or in greenhouses, have been used for larches, but with the objective of accelerating the genetic improvement program for the species (Eysteinsson *et al.* 1993, Danielson and Riley 1995). Sheltered seed orchards have many advantages over conventional seed orchards, including temperature control (thus limiting frost risk, especially in spring), optimization of cultural conditions and the possibility of rapidly renewing the plant material as new selections are made by the genetic improvement program (Nanson 2004). However, the height of the structures used for sheltered orchards rapidly limits the development and flowering of the larch grafts, necessitating regular pruning of the tops of the trees (Eysteinsson *et al.* 1993).

This paper reports on a novel concept for sheltered seed orchards. Our objective was to develop a technique aimed at producing large quantities of HL seeds with a high genetic diversity to meet the needs of the provincial HL reforestation program. To do this, our work focused on i) the culture of EL and JL grafts in containers, to ensure the growth and regular production of both male and female cones; ii) the optimization of a structure that would allow sufficient graft development for abundant and regular cone production, with minimal maintenance, particularly during the winter months; iii) the control of expenditures for constructing the structure as well as for the operating costs; iv) the development of a method for collecting large quantities of high-quality pollen with a minimal workforce, and v) the evaluation of the operational feasibility of the production method with a large inventory of trees.

Materials and Methods

Infrastructure

The sheltered prototype larch seed orchard was constructed in 2000 at the "Centre d'Expérimentation et de Greffage de Duchesnay" (Duchesnay Grafting and Experimenting Center), 30 km north east of Québec City, Quebec, Canada (46° 51′ 05″ N, 71° 38′ 37″ W). The research station belongs to the Direction de la recherche forestière (Forest Research Branch) of the ministère des Ressources naturelles et de la Faune du Québec (Québec Ministry of Natural Resources and Wildlife).

Larch is characterized by rapid juvenile growth. To limit the need for regularly topping the trees, we chose to invert the arches of a conventional tunnel (Harnois, Ovaltech®, width 7 m, arches spaced at 1.5 m), to construct a "cathedral" roof. This increased the height at the centre of the tunnel by 30% (4.6 m rather than 3.5 m at the centre, and 2.6 m at the sides of the tunnel), while reducing the width of the tunnel from 7.7 m to 4.4 m (Fig. 1a). A polyethylene tunnel cover (UVA Overwinter White, Ginegar Plastic Products Ltd, Israel) is installed at the end of October. The cover prevents the accumulation of snow inside the tunnel during the winter months (November to April) and allows pollination operations to begin earlier in the spring (early April). The steepness of the tunnel walls also limits snow accumulation and the need to

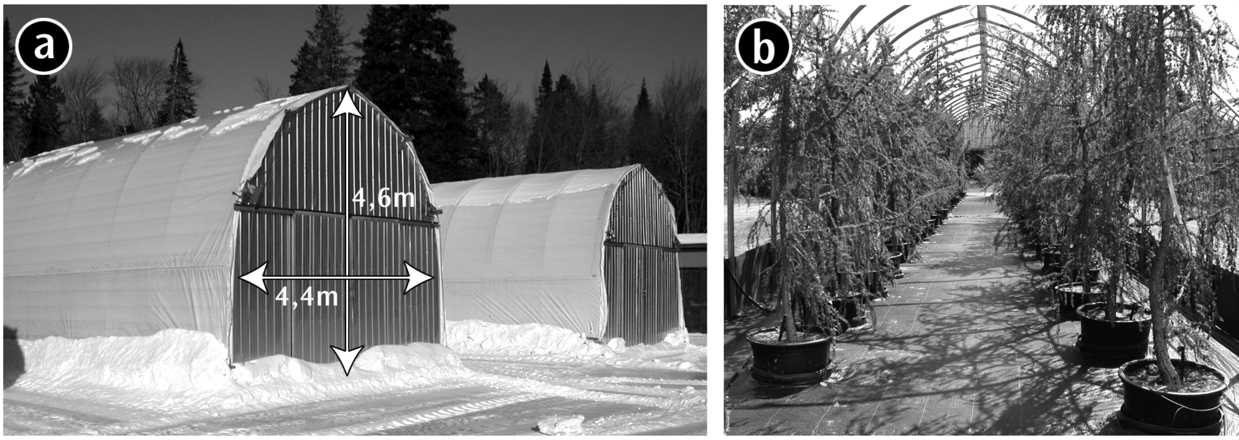

Fig. 1. "Cathedral" tunnel, **a:** the tunnel covers are lowered during the winter. The steep slope of the sides of the tunnel facilitates maintenance and snow removal. **b:** A view of the trees inside the tunnel after the covers have been removed. The pots are staggered to facilitate pollination operations and pollen collection in the spring.

remove it from the structure, an important consideration for northern regions. The tunnel cover is removed after the pollination operations have been completed (Fig. 1 b) and the flowers are closed—at the end of May or the beginning of June. This optimizes exposure to natural sunlight during the growing season.

Plant material and cultural conditions

The EL and JL trees were produced by grafting in 1984, 1986 or 1995. The scions of both species were harvested from clonal plantations as well as from trees selected during testing of descendants of first generation larch improvement programs. In 2004, the trees in the seed orchard were analysed using molecular markers (Acheré *et al.* 2004, Gros-Louis *et al.* 2005) to ensure the purity of both the EL and JL parent trees (clones).

To produce seeds with high genetic diversity, we used 27 EL clones (137 grafts; 1 to 15 ramets/clone) and 30 JL clones (122 grafts; 2 to 13 ramets/clone). This large number of trees enabled us to evaluate the operational feasibility of producing a large quantity of seeds.

The grafts were cultivated in 104-litre pots (Nursery Supplies Inc., Grip Lip, Chambersburg, Pennsylvania, USA) using the pot-in-pot principle. The pots were partially buried in the soil (Fig. 2a). This system allowed the pots to be easily moved, while preventing root penetration into the soil. Burying pots in the soil not only increased the stability of the trees, but also provided an additional height margin of 50 cm, which is not negligible given the rapid growth of the grafted trees.

The trees were placed in staggered rows to optimize available space and incident light (Fig. 1b). Irrigation and fertilization were provided through a drip irrigation system (7-mm exterior tube diameter, pierced with five holes, 2 mm in diameter). To induce air pruning of the roots, the walls of the interior pot were pierced with 158 holes, each 2 cm in diameter (Fig. 2a). The holes were distributed at 3-cm intervals in staggered rows, beginning 12 cm from the bottom of the pot and continuing upwards for 23 cm. In addition, to ensure adequate drainage, the interior pot was elevated slightly on a 2-cm deep layer of gravel (2 cm in diameter) in the bottom of the exterior pot.

The prototype orchard is comprised of five "cathedral" tunnels: two of 60 metres in length, which can each accommodate 77 pots, two of 45 metres in length for 57 pots and one 30-metre tunnel with a capacity of 47 pots. The EL and JL grafted trees are cultivated in different tunnels.

The growing substrate is a mixture of peat, vermiculite and perlite (60%, 20%, 20%, v/v, respectively) as well as small quantities of other components (montmorillonite, brown soil, sand). Granular slow release fertilizers and mineral supplements (Super Phosphate 0-20-0 [200 g[3]], Mini K-Mag 0-0-20-10 [200 g], dolomite [100 g], gypsum [100 g], and Nutricote® 14-14-14, 40 days [70 g] and 18-6-8, 180 days [130 g] [Morton's Horticultural Products Inc., Tennessee, USA]) are also added. During the first two years of growth in the 104-litre pots, the trees are given a top dressing (25 g Nutricote® 14 and 41 g Nutricote® 18) at the beginning of June. In addition, every two weeks between the beginning of June and the end of October, the trees are fertilized with a soluble fertilizer to stimulate growth and provide an adequate nutrient supply for seed development. On average, each tree received 6.5 g nitrogen (N), 9.4 g phosphorus (P) and 14.9 g potassium (K) annually. Flowering was not induced by either hormone treatments or by stem girdling. In addition to the ongoing air pruning of the roots, every two years we also trimmed the root systems, removing approximately 20% of the roots at the bottom of the root ball.

Temperature monitoring inside and outside the "cathedral" tunnels

Probes (Minilog 8 bit, Vemco, Shad Bay, Canada) were installed to monitor air temperature in the "cathedral" tunnels throughout the year. The data were recorded every half hour to determine the maximum, minimum and average temperatures on a daily basis. For comparison purposes, a temperature probe was placed outside, 200 m from the tunnels. Temperature data were compiled between 2001 and 2005.

[3]The values presented represent the mass applied to each 104-litre pot.

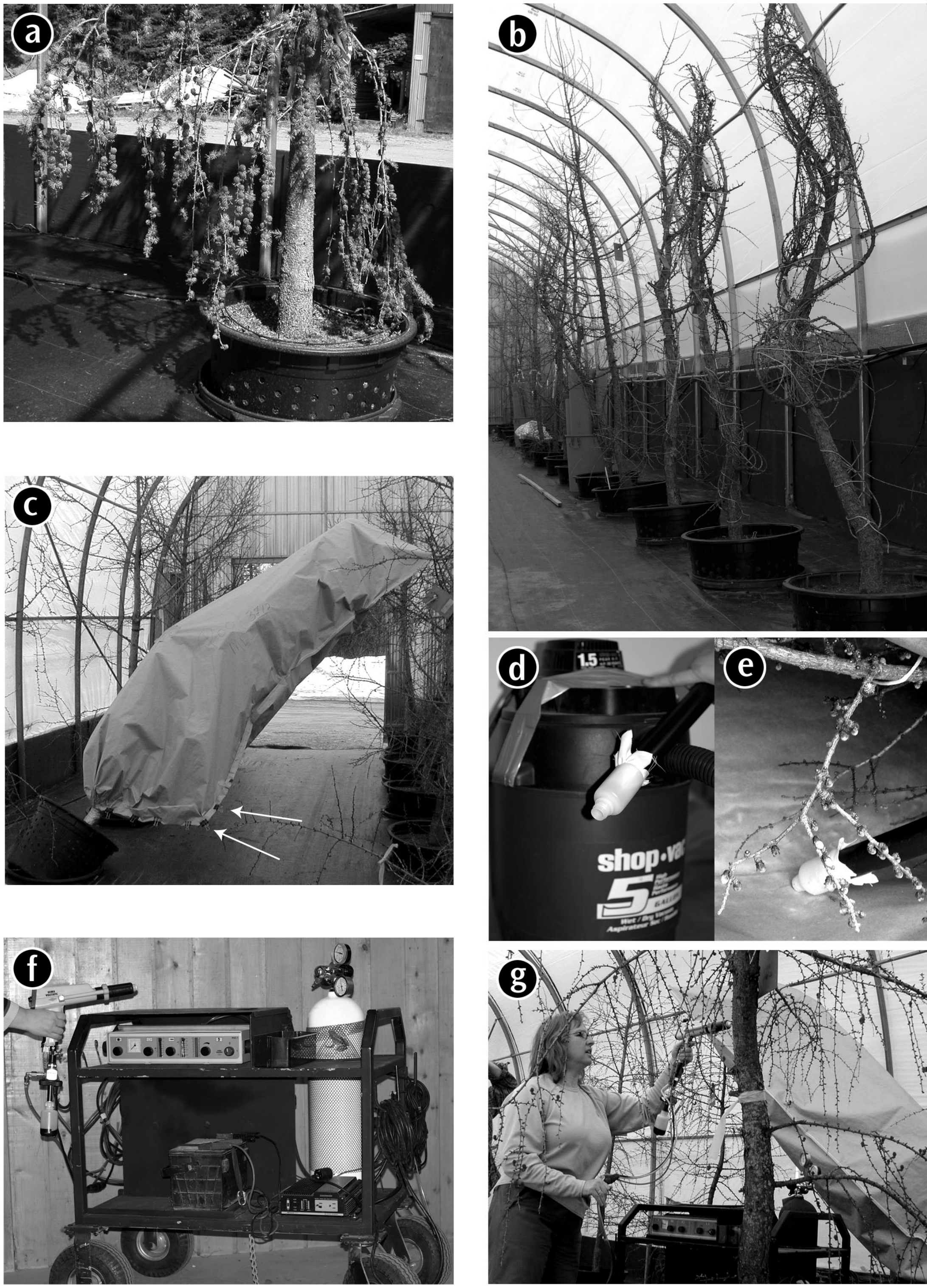

Fig. 2. a: Larch grafts are cultivated using in a pot-in-pot system. The interior pot is pierced to encourage air pruning of the root system. A layer of crushed rock is placed in the bottom of the exterior pot to improve drainage; **b:** Grafts, installed in an operational seed orchard at the Berthier forest nursery. Note that the branches are tied together to facilitate crown bagging for pollen collection; **c:** A *Larix kaempferi* graft whose crown has been encased in a bag in preparation for pollen collection. The tree has been placed at an incline to facilitate pollen deposition. The opening on the side of the bag, through which the pollen will later be collected, is held closed with clamps (at arrows); **d:** Vacuum system and nozzle used for pollen collection. The pollen accumulates in a bottle that is emptied and cleaned after collecting pollen from each clone; **e:** During dissemination, the pollen falls to the bottom of the bag and is collected with a vacuum; **f:** A cart designed for transporting the electrostatic pistol and other pollination equipment (bottle of compressed air, power source, electrical transformer and air filter); **g:** Pollination using the portable electrostatic pistol.

Seed production

Selection of the grafts

At the beginning of spring, when the flowers are easily recognizable on the trees, we conduct an exhaustive flowering inventory to determine which grafts are suitable for pollen collection and which ones can be pollinated. To serve as a pollen source, the graft has to have a minimum of 300 male flowers. To be pollinated, the graft must bear at least 100 female flowers. However, this selection also takes into account the amount of pollen reserves available.

To facilitate operations, the grafts destined to be pollinated are moved before their flowers become receptive (mature). They are grouped, by species, in separate tunnels and distanced from the trees from which the pollen is to be collected, thus avoiding the possibility of pollen contamination. The grafts that are not scheduled for pollen collection or pollination are either emasculated by removing all male flowers, or moved outside the tunnel to prevent pollen contamination of the target trees.

Pollen collection and treatment

The pollen collection bags are individually prepared from Kraft paper (1.5 m wide, 0.15 mm thick; Unisource, DD60, Québec, Canada). The length of the bag is adapted to the size of the selected tree crowns. The side seams are stitched, leaving an opening in the bottom to allow pollen collection. The branches of the pollen-producing grafts are tied together (Fig. 2b). A bag is then placed over the tree crown and tied around the base of the trunk. Clamps are used to hold the opening on the side of the bag closed. The potted tree is then set at an angle to permit the pollen to fall naturally to the bottom of the bag (Fig. 2c).

The pollen from each clone is collected separately. The process is repeated twice for each tree. The first collection is made two days after the beginning of pollen dissemination and the second collection, three or four days later, up to the end of the dissemination period. This interval between the two collections may be modified depending on the prevailing meteorological conditions. Warm temperatures (> 15°C) outside the tunnel accelerate pollen dissemination. Rain or high relative humidity necessitates rapid collection of the pollen that has fallen into the bags because moisture reduces pollen viability. The pollen is collected using a vacuum (Shop-Vac Corporation, Williamsport, Pennsylvania, USA), whose outlet has been blocked with a dense fabric, and a bottle in which pollen accumulates (Fig. 2d, e). To avoid contamination between pollen lots, the vacuum nozzle is carefully cleaned after collecting pollen from each clone. The pollen is sifted through a screen (VWR, 120 mesh, 20 cm diameter, 5 cm high) to eliminate impurities. It may then be immediately used for pollination or be prepared for long-term storage in the pollen bank of the ministère des Ressources naturelles et de la Faune du Québec (Colas and Mercier 2000). The pollen collection bags are removed when pollen dissemination is complete.

Before storage, the pollen is dried in a mechanical convection oven (Precision Scientific, Winchester, USA) for four hours at 40°C (Colas and Mercier 2000). Its viability is evaluated by measuring the electrolytical conductivity (Ching and Ching 1976) of a filtrate prepared from pollen that has been rehydrated for 16 hours (Webber and Ross 1995). In agreement with Baldet (2006), our preliminary studies determined that the measured electrolytical conductivity should be less than 20 $\mu S \cdot cm^{-1}$ if the pollen is to be used in the near future or stored in the pollen bank. The water content of the pollen is determined using the procedure described by Webber (1991). The pollen is vacuum sealed into bottles or polyethylene bags and kept in a freezer at -25°C (Colas and Mercier 2000). Depending on the volume collected, the pollen from each clone is either conserved separately or mixed with that of other clones. In the latter case, the volume of pollen from each clone in the mixture is recorded.

Pollination

The grafts selected for pollination are carefully emasculated, leaving only female flowers on the trees. These flowers are not protected by pollination bags. The selected pollen is applied using a portable electrostatic pistol (Gema Voltstatic model 11024, ITW Gema, Indianapolis, USA) adapted from the model developed by Philippe and Baldet (1997). The pistol and its accompanying equipment are attached to a cart designed by the Direction de la recherche forestière to make easy rapid moves within the tunnel (Fig. 2f). Each graft is pollinated twice, at a two-day interval during the period in which the female flowers are most receptive (Fig. 2g). Thus, depending on the species and the year (2001 to 2005), flowers receptivity and pollination date varied between April 20 and May 5. The volume of pollen applied on each graft (3 to 8 ml) depends on the number of female flowers.

After the flowers have closed, the grafts are returned to their original place in the tunnels. As soon as the risk of spring frost has passed, the tunnel covers are removed and fertilization can begin. Cones are harvested about four or five months after pollination. The total number of cones is determined for each species.

Seed processing

Cones of each species (EL and JL) are kiln-dried separately at 60°C for 16 hours. For dewinging, seeds are soaked 16 hours, dried 24 hours at 30°C, and then dewinged manually by rubbing. Empty seeds are eliminated with a gravity separator. Numbers of filled and empty seeds are determined with a seed counter (801 Count-A-Pack seed counter, Seedburo, Chicago, USA).

Evaluation of seed germination under artificial conditions

Germination tests are done in a germinator (model G30, Conviron, Winnipeg, Canada) during the winter following the pollination season. The tests are conducted following ISTA norms (1999) for JL: depending on the size of the seed lots, four replicates of 100 seeds (lots ≥ 5000 seeds) or four replicates of 50 seeds (lots < 5000 seeds) are used. The seeds are placed on a cellulose substrate (Kimpak Seedburo, Chicago, USA) cut to fit the germination plate, moistened with 250 ml of deionized water and placed in a transparent container adapted by Wang and Ackerman (1983) (Spencer Lemaire, Alberta, Canada). The seeds are randomly distributed in the germination plates in four sub-samples, and placed in the refrigerator (4°C) for 21 days. At the end of the stratification period, the germination plates are placed in the germinator for 21 days (alternating day/night temperatures 30/20°C; 16-hour days; 85% relative humidity). A seed is considered to have germinated once the cotyledons are visible (stage 2 as described by Wang [1973]). After each tally, the seedlings are removed from the plate to facilitate subsequent tallies and avoid counting errors. The germination percentage is validated against the acceptable range specified by ISTA (1999).

Results

Temperature monitoring

The average, maximum and minimum temperatures observed in the "cathedral" tunnels between the months of November and May for the study period (2001 to 2005) were higher than those observed 200 m outside the tunnels (Fig. 3a, b). For the five years, the maximum temperatures inside the "cathedral" tunnels were above 0°C as of January 25, whereas outside the tunnels maximum temperatures did not reach 0°C until March 9. Average daily temperatures inside and outside the tunnels during the same period were above freezing beginning on March 17 and April 7, respectively. With respect to average minimum temperatures, values were positive as of May 9 inside the "cathedral" tunnels, and May 13 outside the tunnels. These average minimum temperatures were not cold enough to provoke frost damage on female flowers that were already pollinated. The temperature differences observed between inside and outside illustrate the greenhouse effect created inside the tunnel. More precisely, Fig. 4 shows the temperature typically observed between January and April. The maximum temperatures inside the tunnel are much higher than the maximum temperatures outside the tunnel between mid-February

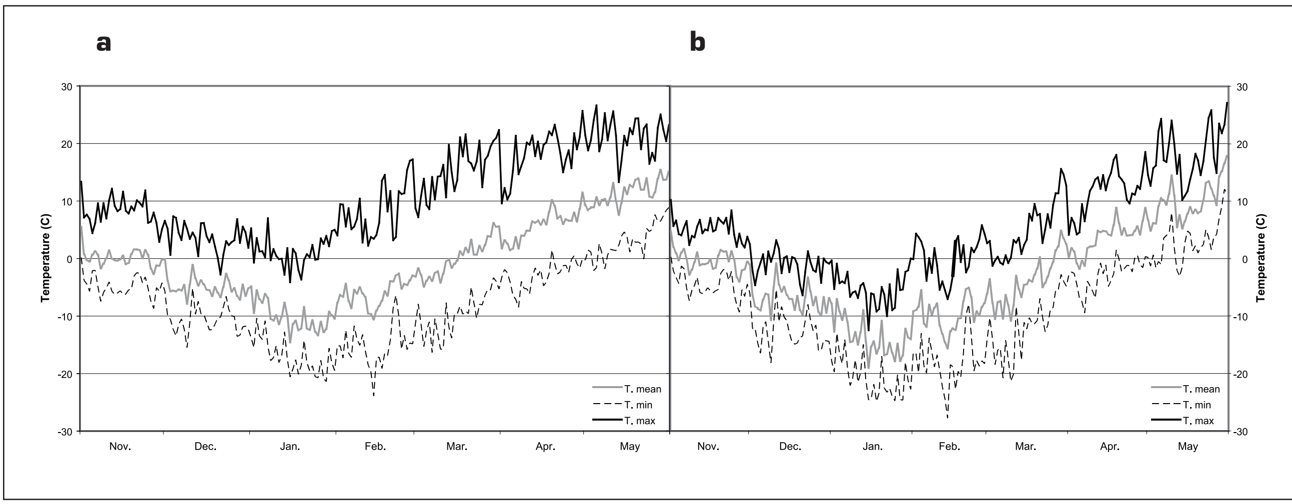

Fig. 3. Average mean, minimum and maximum temperatures observed between the months of November and May (2001 to 2005). **a:** Temperature observed inside a "cathedral" tunnel; **b:** Exterior temperature, observed 200 m from the tunnel.

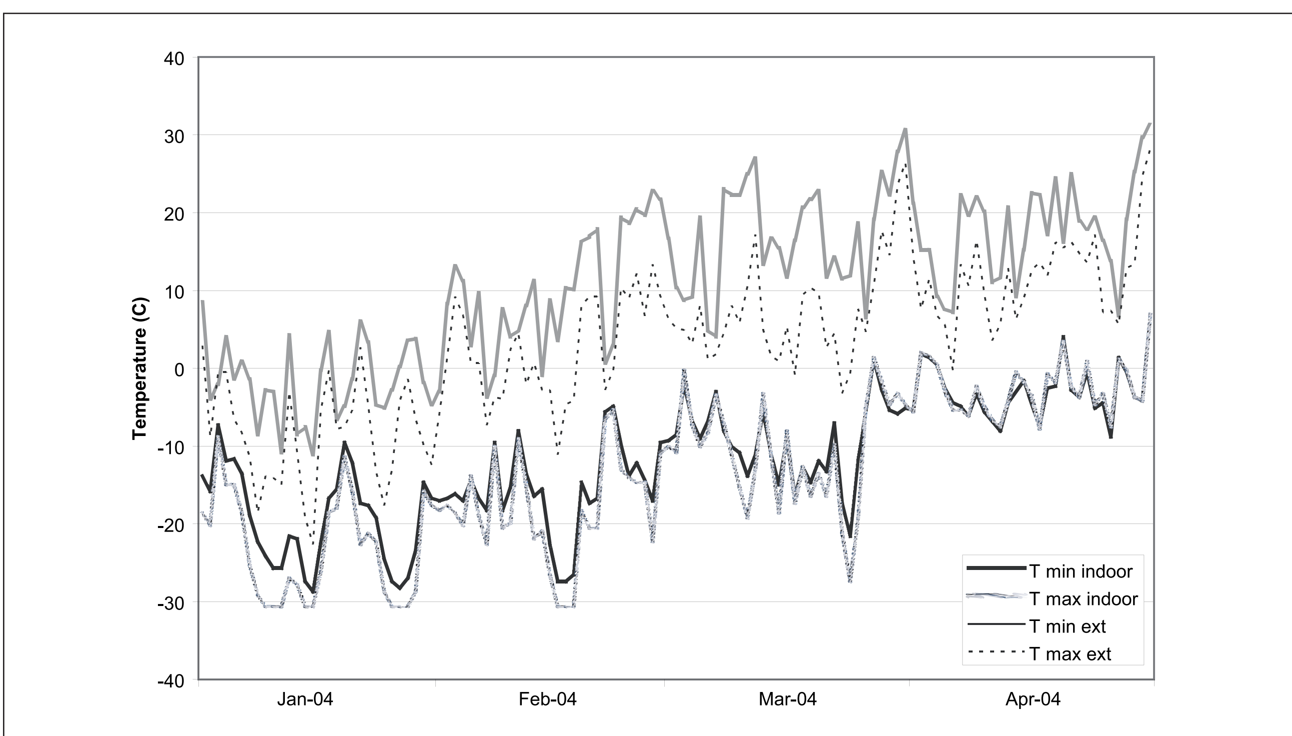

Fig. 4. Minimum and maximum temperatures observed inside, and 200 m outside, a "cathedral" tunnel between January 1 and April 30, 2004.

Table 1. Percentage of Japanese Larch (JL) and European Larch (EL) clones, cultivated in "cathedral" tunnels, which bore female or male flowers during the study period (2001 to 2005). There were 30 and 27 clones present in the seed orchard for JL and EL, respectively. (♀ = female, ♂ = male)

	Clones bearing ♀ flowers		Clones bearing ♂ flowers		Clones bearing both ♀ and ♂ flowers	
Year	JL	EL	JL	EL	JL	EL
2001	3%	33%	63%	19%	0%	4%
2002	40%	37%	17%	37%	3%	11%
2003	67%	33%	87%	30%	63%	26%
2004	67%	56%	70%	44%	60%	37%
2005	67%	63%	50%	26%	50%	26%

Table 2. Percentage of JL and EL ramets, cultivated in "cathedral" tunnels, that were pollinated or from which pollen was collected between 2003 and 2005. To be selected for pollen collection or pollination, the grafts had to possess a minimum of 300 male flowers or 100 female flowers, respectively. There were a total of 122 ramets for 30 clones of JL, and 137 ramets for 27 clones of EL.

	% pollinated ramets		% ramets used for pollen collection	
Year	JL	EL	JL	EL
2003	16%	20%	23%	5%
2004	18%	14%	8%	14%
2005	22%	33%	17%	12%

Table 3. Number of pollinated cones harvested, by species and by year, and the weighted number of filled seeds per cone for the 2003 to 2005 pollination campaigns for Japanese Larch (JL) and European Larch (EL). Germination of stratified seed was evaluated under both artificial (germinator) and nursery conditions. For the latter, only the data for the 2003 seed lots are presented, as the 2004 and 2005 seeds have not yet been used for seedling production.

Species of mother tree	Year	Number of cones harvested	Filled seeds per cone (Weighted average)	Germination (%) Germinator	Germination (%) Nursery
JL	2003	12 912	4.9	74	78
	2004	5 605	10.9	68	–
	2005	5 742	5.9	85	–
EL	2003	17 373	3.4	79	71
	2004	2 492	19.5	68	–
	2005	9 900	3.5	64	–

and mid-March, as a result of the greenhouse effect. During the same period, the minimum temperatures inside and outside the tunnel are very similar.

Elevated temperatures inside the tunnel accelerate flower development. Flowers inside the tunnel were, phenologically, two to three weeks more advanced than those on larch trees outside the tunnel (data not presented). This phenomenon is due to the observed temperature differences inside and outside the tunnels. The earlier flowering of the trees inside the tunnels eliminates all risks of contamination from pollen produced on trees outside the tunnels. In addition, the higher temperatures inside the tunnels effectively protect the flowers against commonly-occurring late spring frosts.

Flowering

Both male and female flowering were regular for either species between 2001 and 2005 (Table 1). As a result of the abundant flowering over the study period, we were able to conduct a large number of pollinations and consequently, produce seeds with high genetic diversity. In general, a larger proportion of the JL clones bore flowers than did the EL clones. However, the number of clones bearing flowers varied among the five study years. In 2001 and 2002, low percentages of JL and EL clones bore both male and female flowers (< 11%). In contrast, between 2003 and 2005, 26% to 37% of the EL clones bore both types of flowers, and at least 50% of JL clones did the same.

An equivalent number of pollinations was performed for both species between 2003 and 2005 (Table 2). However, the pollination of the JL grafts was often limited by the quantity of EL pollen available, due to a poorer male flowering of EL clones. The percentage of EL ramets from which we collected pollen never exceeded 14% between 2003 and 2005.

Pollen collection

The quantity of pollen collected from each graft sometimes exceeded 15 ml, but depended on both the number of flowers and the species. The total volume of pollen collected from an individual species varied from year to year. Between 2001 and 2005, the volume of JL pollen collected varied between 60 and 420 ml. For EL, the volume varied between 50 and 380 ml. Since 2001, the viability of the pollen lots collected by crown-bagging conforms to the established quality standards, electrolytical conductivity values ranging from 3.9 to 14.5 $\mu S \cdot cm^{-1}$.

Seed production

The production of filled seed was variable, as was the seed yield per cone, depending on the species of the mother tree and the pollination year (Table 3). Between 2003 and 2005, the yield for JL ranged from 4.9 to 10.9 seeds per cone, whereas EL cones contained between 3.4 and 19.5 seeds. The germination percentage for the seed lots under artificial conditions was 64% to 85% (Table 3).

Discussion

This research study, conducted to put in place a novel system for producing HL seeds under northern climatic conditions, has led to significant progress with respect to the volume, cost and genetic diversity of the seeds produced.

The establishment of sheltered seed orchards is an avenue that has been explored for many species (Ross 1988, Eysteinsson *et al.* 1993, Mercier and Périnet 1998). The advantage of these orchards lies in the optimization of growth and flowering conditions. However, installation costs and difficulties associated with growing large trees in containers usually limits the development of these types of structures (Philippe *et al.* 2006). We consider that sheltered growing conditions and the pot-in-pot system are particularly appropriate for the production of HL seeds. The modifications made to increase the height of conventional tunnels without incurring substantial additional costs, as well as partially burying the pots in the ground, effectively accommodate the height of the trees. The greenhouse effect created in the tunnel during spring accelerates flower development, with respect to trees grown in unsheltered conditions. It also prevents the possibility of pollen contamination from trees grown outside the tunnel. Growing grafts under tunnel conditions not only protects the pollinated flowers, but also protects the trees from late spring frosts, which are a common occurrence in northern regions.

Before the development of indoor seed orchards, HL seeds were produced outdoors via controlled crossings. However, large-scale controlled crossings were highly impractical in northern regions, because of late frosts and the reduced site accessibility in spring that restrains the use of a lift to reach the flowers. It has been abandoned in Québec for large-scale larch seed production, due to excessive costs for too uncertain results.

The pot-in-pot system permits the trees to be easily moved, facilitates the grouping of trees used for pollen collection or pollination, simplifies management operations in the spring and reduces the risk of pollen contamination. In addition, because the tunnel covers can be removed during the period between pollination and cone collection, summer temperatures in the tunnels are not excessive and there is no need for ventilation or air conditioning. The absence of tunnel covers also gives the trees access to full sunlight, which enhances flower induction and seed development (Chandler 1967, Shearer and Schmidt 1987, Uchiyama *et al.* 2004). The effect of light, in combination with the fertilization regime and air pruning of the roots, result in abundant and recurring flowering of the grafts.

Larch pollen is usually collected manually. A large workforce has to be mobilized over a short period of time to harvest the volumes of pollen required for pollination treatments. Because the flowers are distributed throughout the crown, the process is very time-consuming. In addition, the flowers of the same species disseminate their pollen during the same period, making it difficult to avoid contamination among pollen lots. The collection method developed by Eysteinsson *et al.* (1993) cannot guarantee the absence of contamination, since pollen is collected at the time of dissemination in open reservoirs placed on the branches containing male flowers.

Philippe *et al.* (2001) consider that pollen collection is the major investment in all the processes of seed production, and that rain or frost can rapidly ruin all the work. We have developed a method of bagging tree crowns to collect pollen, with several advantages which make it very cost-effective:

- It is not affected by wind or precipitation. Dissemination can continue even when it is raining, and pollen will not be washed out of the cones during the process.
- There is no contamination between pollen lots. Because the pollen falls into a bag, it is not transported by air throughout the tunnel. The precautions taken during pollen collection minimize the risk of pollen contamination.
- Only the naturally disseminated and therefore, highest quality, pollen is collected (Beers *et al.* 1981). The collection of low-quality, immature pollen is avoided. Also, by collecting only disseminated pollen, the extraction step is eliminated.
- All of the available pollen produced on the graft is collected, regardless of the environmental conditions.
- Pollen can be collected from a large number of clones. Therefore, the pollen reserve has a high genetic diversity.
- Only a minimal amount of pollen (< 5%) is lost due to adhesion to the pollen collection bag. This quantity is very low, compared to the 50% to 60% value cited by Philippe *et al.* (1993).
- The size of the collection bag is adapted to the size of each of the individual grafts.
- The workforce requirement is significantly reduced. Pollen collection can usually be done by two people.

The pollination method used in sheltered larch seed orchards permits a large number of different crosses to be conducted, thus guaranteeing a large quantity of hybrid seeds with a high genetic diversity. In comparison with other hybrid larch seed production techniques (Eysteinsson *et al.* 1993, Tröber and Haasemann 2000, Philippe *et al.* 2001), the one reported here allows the use of a large number of clones for both parental species (EL and JL).

The yield of filled seeds per cone that we obtained varied between years and among types of cross (EL × JL or JL × EL). The filled seed yields we have are similar to those obtained by Webber et Ross (1995), but are inferior to those presented by Philippe *et al.* (2006). It is important to note that in the latter study, a single clone of EL was used as the female parent, which may explain a better fecundity of the crossings.

The pollen we used for the pollinations was of excellent quality, based on the conductivity results. Temperature conditions were about the same in the three years, as were the pollination periods. Therefore, we assume that the yearly variations of seed yield are due to differences between individuals.

We did not evaluate the fecundity of each clone because we collected cones in bulk, regardless of mother clones. In the tunnels we have clones of different ages (grafted in 1984, 1986 and 1995). For both JL and EL, we observed that the production of male and female flowers diminished with graft age after the grafts were 15 years old (data not presented). This observation has also been made for Tamarack (*Larix laricina* [Du Roi] K. Koch) (Eysteinsson and Greenwood 1993). In 2004, for example, the proportion of old grafts pollinated was the lowest and we obtained the highest filled seed yield of the three years of study. The proportion of older grafts that are pollinated depends on the year, and we assume that it could explain the differences in seed yield.

The germination rates evaluated under artificial conditions for the HL seeds produced in our prototype seed orchard were between 64% and 85%. These values are similar to those obtained by Bonnet-Masimbert *et al.* (1998). The seed lots produced in 2003 were also evaluated under nursery conditions. Their germination rate was equivalent to that obtained under artificial conditions.

In order to appraise the economic performance of our HL seed production system, we evaluated our seed production costs, albeit at the research scale. Since we used existing infrastructures, only labour costs were included in the calculations. Results were expressed in terms of viable HL seeds, by taking into account total number of cones harvested, proportion of filled seed per cone and germination rates. Seed production costs varied between years from 0.24 to 0.45 $ (CAD) per viable HL seed. Fluctuations are mainly attributed to variations in flowering abundance. We expect that transfer to the operational scale will further reduce production costs. In the United Kingdom, Perks *et al.* (2006) reported HL seed production costs at more than 1 £ (GBP), or about 2 $ (CAD) per viable seed. However, their method of calculation was not detailed and prevents any direct comparisons.

To maintain the orchard productivity, it is necessary to periodically renew the plant material grown in the tunnels. A period of 15 years corresponds, approximately, to the delay between two genetic selection cycles. The new material will therefore be a product of the new recommendations of the genetic improvement program and will permit rapid production of better-quality plants for reforestation programs.

After five years of development, we have demonstrated that the production of HL seeds in a sheltered seed orchard is operationally feasible. This method has now been implemented at the Berthier forest nursery (Lanaudière region, Québec). The first pollen collection and pollination operation took place in the spring of 2006, on grafts representing the best material selected by the genetic improvement program for larch, 20 JL and 20 EL from more than 40 000 candidate trees (Perron 2006). The results obtained with respect to the number of crossings conducted, the number of seeds produced and their germination characteristics meet the demand for seeds and reforestation stock. The seedlings grown from the HL seeds produced at Berthier will be of the highest genetic quality available in the province of Québec. They will serve as stock-plants for mass production of HL cuttings.

Conclusion

Larches present numerous difficulties for seed production. Cultivating trees in containers under sheltered conditions has shown its potential for regular flowering of both male and female flowers on JL and EL grafts. The pollen collection and pollination methods reported in this study yield large quantities of HL seeds with a high genetic diversity. These seeds will meet the growing need for the production of HL seedlings, a demand that will only increase as ligniculture develops across Canada and elsewhere.

Acknowledgements

The present study was initiated in collaboration with Stéphan Mercier and Ante Stipanicic (Direction de la recherche forestière). We also acknowledge the technical contribution and tips of Nicole Robert, Martine Lapointe, Gaston Lapointe, Jean-Philippe Mottard and Fernand Gosselin, as well as the staff at the Centre d'expérimentation et de greffage de Duchesnay. The editorial comments and suggestions of Dr. Mohammed S. Lamhamedi and Dr. Robert Jobidon were well appreciated. We also express our thanks to Debra Christiansen-Stowe for manuscript translation and to two anonymous reviewers. This project was funded by the Direction de la recherche forestière (#0400-3117), and also received financial support from the Direction générale des pépinières et des stations piscicoles, both part of the Ministère des Ressources naturelles et de la Faune du Québec.

References

Acheré, V., P. Faivre-Rampant, L.E. Pâques and D. Prat. 2004. Chloroplast and mitochondrial molecular tests identify European × Japanese larch hybrids. Theor. Appl. Genet. 108(8): 1643–1649.

Baldet, P. 2006. Fiches de recommandations pour la technologie du pollen. *In* Cemagref (ed.). Reproduction sexuée des conifères et production de semences en vergers à graines, pp. 371–396. Cemagref, Collection Synthèses, Paris.

Beers, W.L., J. Bivens and J.E. Mocha. 1981. Pollen collection. *In* E.C. Franklin (ed.). Pollen Management Handbook. pp. 30–32. USDA, Forest Service, Washington, DC.

Bolghari, H.A. and V. Bertrand. 1984. Tables préliminaires de production des principales essences résineuses plantées dans la partie centrale du Sud du Québec. Service de la recherche (Terres et Forêts), Ministère de l'Énergie et des Ressources. Mémoire de recherche forestière 79. 392 p.

Bonnet-Masimbert, M., P. Baldet, L.E. Pâques and G. Philippe. 1998. From flowering to artificial pollination in larch for breeding and seed orchard production. For. Chron. 74 (2): 195–202.

Bramlett, D.L. and C.H. O'Gwynn. 1981. Controlled pollination. *In* E.C. Franklin (ed.). Pollen Management Handbook. pp. 44–51, USDA Forest Service, Washington, DC.

Chandler, C. 1967. A progress report of the larch improvement program at Boyce Thompson Institute. Contributions from Boyce Thompson Institute 23(9): 319–326.

Ching, T.M. and K.K. Ching. 1976. Rapid viability tests and aging study of some coniferous pollen. Can. J. For. Res. 6: 516–522.

Chui, Y.H. and G. MacKinnon-Peters. 1995. Wood properties of exotic larch grown in eastern Canada and north-eastern United States. For. Chron. 71(5): 639–646.

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

Danielson, J. and L. Riley. 1995. Western larch containerized seed orchards: adapting a concept to meet the production seed needs of the Pacific Northwest. *In* W.C. Schmidt and K.J. McDonald (eds.). International Symposium on Ecology and Management of *Larix* forests: a look ahead. Whitefish, Montana, 5–9 October 1995. pp. 478–481.

Eysteinsson, T. and M.S. Greenwood. 1993. Effects of maturation and gibberellin $A_{4/7}$ on flowering and branching characteristics of *Larix laricina.* Can. J. For. Res. 23(1): 14–20.

Eysteinsson, T., M.S. Greenwood and J. Weber. 1993. Management of a prototype indoor orchard for accelerated breeding of larch. College of Forest Resources, Maine Agricultural Experiment Station, University of Maine. 18 p.

Gros-Louis, M.-C., J. Bousquet, L.E. Pâques and N. Isabel. 2005. Species-diagnostic markers in *Larix* spp. based on RAPDs and nuclear, cpDNA, and mtDNA gene sequences, and their phylogenetic implications. Tree Genet. Genomes 1(2): 50–63.

Häcker, M. and F. Bergmann. 1991. The proportion of hybrids in seed from a seed orchard composed of two larch species (*L. europaea* and *L. leptolepis*). Ann. Sci. Forest. 48: 631–640.

ISTA. 1999. International rules for seed testing 1999. Seed Sci. & Technol. 27 (Supplement 1): 1–362.
Kosinski, G. 1986. Megagametogenesis, fertilization, and embryo development in *Larix decidua*. Can. J. For. Res. 16: 1301–1309.
Kuromaru, M., T. Oshima, K. Kita and K. Uchiyama. 2004. Potentialities of the new designed seed orchard for the hybrid larch (*Larix gmelinii* × *L. leptolepis*) F_1. *In* IUFRO International Symposium Larix 2004 – IUFRO Working Party S2.02-07 Larch Breeding and Genetic Resources. Kyoto and Nagano (Japan) 26 Sept. – 1 Oct. 2004. p. 26.
Lee, S. 2003. Breeding hybrid larch in Britain. Forestry Commission, Edinburgh. Information Note 52. 4 p.
Mercier, S. and P. Périnet. 1998. The second generation seed orchard research project at the Direction de la recherche forestière in Québec. For. Chron. 74(2): 181–184.
Nanson, A. 2004. Génétique et amélioration des arbres forestiers. Les Presses Agronomiques de Gembloux, Gembloux (Belgique). 712 p.
Owens, J.N. and M. Molder. 1979. Sexual reproduction of *Larix occidentalis*. Can. J. Bot. 57: 2673–2690.
Pâques, L.E., G. Philippe and D. Prat. 2006. Identification of European and Japanese larch and their interspecific hybrid with morphological markers: application to young seedlings. Silvae Genetica 55(3): 123–134.
Perks, M., A. Harrison, H. Mc Kay and J. Morgan. 2006. An update on nursery propagation and establishment best practice for larch in Britain. Forestry Commission. Information Note 080. 6 p.
Perron, M. 2006. Quality and productivity of our future hybrid larch: Spin-offs from 35 years of larch breeding at the MRNF-Québec. *In* Colloque Eastern CANUSA. Les sciences forestières audelà des frontières / Eastern CANUSA Conference. Forest Science across the Borders. Université Laval, Québec (Canada), October 19–21, 2006. p. 122.
Philippe, G. and P. Baldet. 1997. Electrostatic dusting : an efficient technique of pollination in larch. Ann. Sci. Forest. 54: 301–310.
Philippe, G., P. Baldet and B. Héois. 2001. Mélèze hybride: les premiers fruits d'un mariage heureux. *In*: Riou-Nivert, Philippe (Ed.), Le mélèze. pp. 28–33. Institut pour le développement forestier, Paris.
Philippe, G., P. Baldet, B. Héois and C. Ginisty. 2006. Reproduction sexuée des conifères et production de semences en vergers à graines. Cemagref, Collection Synthèses. Paris. 570 p.
Philippe, G., M. Cazet, P. Baldet, J.D. Boye, A. Valadon and V. Bourlon. 1993. Accélération de la production de matériel "mélèze hybride," 2ème tranche : rapport d'activité 1992. CEMAGREF. 28 p.
Ross, S.D. 1988. Effects of temperature, drought, and gibberellin $A_{4/7}$, and timing of treatment, on flowering in potted *Picea engelmannii* and *Picea glauca* grafts. Can. J. For. Res. 18: 163–171.
Rudolf, P.O. 1974. *Larix* Mill. Larch. *In* Seeds of woody plants in the United States. pp. 478–485. USDA, Forest Service, Washington, DC.
Shearer, R.C. and W.C. Schmidt. 1987. Cone production and stand density in young *Larix occidentalis*. For. Ecol. Manage. 19(1–4): 219–226.
Slobodnik, B. and H. Guttenberger. 2000. Ovule, megaspores, and female gametophyte formation in *Larix decidua* Mill (Pinaceae). Acta Biologica Cracoviensia Series Botanica 42(2): 93–100.
Slobodnik, B. and H. Guttenberger. 2005. Zygotic embryogenesis and empty seed formation in European larch (*Larix decidua* Mill.). Ann. For. Sci. 62(2): 129–134.
Stipanicic, A. 1999. Les programmes d'amélioration génétique : bilan des réalisations. Les mélèzes laricin et hybrides. *In* Service Canadien des Forêts (CFL), Forêt Québec (DRF). (eds.). L'amélioration génétique en foresterie: où en sommes-nous? Rivière-du-Loup, Québec, 28–30 sept. 1999. pp. 77–85.
Tousignant, D. and M. Rioux. 2002. Le bouturage des résineux à la pépinière de Saint-Modeste (Québec, Canada) : 10 ans de recherche, de développement et d'innovations. *In* Troisième rencontre du groupe de la Ste Catherine, 22–24 novembre 2000. Orléans (France). pp. 65–86.
Tousignant, D. and A. Stipanicic. 2000a. *Larix* × *marschlinsii* – Outline data sheet. *In* Forestry Compendium Global Module (Encyclopédie multimédia sur CD-ROM). CABI. 30 p.
Tousignant, D. and A. Stipanicic. 2000b. *Larix kaempferi* – Full data sheet. *In* Forestry Compendium Global Module (Encyclopédie multimédia sur CD-ROM). CABI. 10 p.
Tousignant, D. and A. Stipanicic. 2000c. *Larix decidua* – Full data sheet. *In* Forestry Compendium Global Module (Encyclopédie multimédia sur CD-ROM). CABI. 30 p.
Tröber, U. and W. Haasemann. 2000. Pollination effects in a larch hybrid seed orchard. For. Gen. 7(1): 77–82.
Uchiyama, K., M. Kuromaru and K. Kita. 2004. Climatic factors controlling female flowering of *Larix gmelinii var. japonica* in Northern Japan. *In* IUFRO International Symposium Larix 2004 – IUFRO Working Party S2.02-07. Larch Breeding and Genetic Resources. Kyoto and Nagano (Japan) 26 Sept. – 1 Oct. 2004. p. 25.
Wang, B.S.P. 1973. Laboratory germination criteria for red pine (*Pinus resinosa* Ait.) seed. Proc. Assoc. Off. Seed Anal. 63: 94–101.
Wang, B.S.P. and F. Ackerman. 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.
Webber, J.E. 1991. Interior spruce pollen management manual. B.C. Ministry of Forests. Land management report 70. 25 p.
Webber, J.E. and S.D. Ross. 1995. Flower induction and pollen viability for western larch. *In* W.C. Schmidt and K.J. McDonald (eds.). International Symposium on Ecology and Management of *Larix* forests: a look ahead. Whitefish, Montana, 5–9 Oct. 1992. pp. 395–402.