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5. Eyeing Emergence: Modified Treatments for Terminating Dormancy of Conifer Seeds

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Abstract

Many seeds of coniferous species display a deep primary dormancy at maturity and require several weeks of pre-treatment to produce seed populations that germinate in a vigorous and timely manner. Facilitating an efficient transition from dormancy to germination by devising improved protocols for dormancy breakage is not only important to conifer seed research, aiding in the study of the dormancy process itself, but is also of interest and applicability to commercial forest nursery operations. In the forests of British Columbia, Canada, several conifer species are well adapted to their environment, with seeds needing to experience long durations in the moist state at cool or fluctuating temperatures. These include yellow-cedar (*Callitropsis nootkatensis*), western white pine (*Pinus monticola*), and true fir species such as Pacific silver fir and subalpine

fir (*Abies amabilis* and *A. lasiocarpa*, respectively). The development of dormancy-breaking protocols for the aforementioned species in our laboratory has centred on balancing several key aspects of the method: (1) reducing the time needed to terminate dormancy in the seed population, (2) synchronicity of germination, (3) ease of use, (4) cost effectiveness, and (5) repeatability. For yellow-cedar, the modified protocols were further tested in relationship to promoting vigorous growth in a forest nursery greenhouse setting and after planting at natural stands. This book chapter will review and summarize the modified dormancy-breaking protocols for yellow-cedar, western white pine, and the true firs. Based on the five criteria listed above, very significant improvements compared to traditional dormancy-breaking methods were achieved for these conifer species. For yellow-cedar the modified dormancy-breaking treatments resulted in vigorous growth in the greenhouse and after planting at natural stands.

Key Words: Seed dormancy, conifers, moist chilling, traditional dormancy-breaking protocols, modified dormancy-breaking protocols, alcohols, gibberellic acid, solid matrix priming, true firs (*Abies* spp.), yellow-cedar, western white pine.

1. Introduction

Seed dormancy, an adaptive trait that facilitates the distribution of seed through the dimensions of time and space, has been a key trait in the evolution of both angiosperm and gymnosperm species – helping plants maximize their fitness through successful establishment of subsequent generations ***(1,2)***. The capacity for the imbibed seed to modulate its developmental schedule to

germinate only when environment signals predict that growth and seedling establishment will be successful is a remarkable phenomenon. However, when seeds fall into human hands for use as cultivated crops or in reforestation efforts, dormancy becomes an unwanted and undesirable trait since the grower can usually ensure that conditions in cultivated fields or in the greenhouse are optimal for seedling establishment and plant growth. It is not surprising then that domestication of a crop almost always removes or significantly reduces seed dormancy ***(3,4)***. However this has not been the case for tree species used in reforestation efforts. Despite the development of a more comprehensive silvicultural program, as seen in tree seed orchards and seedling nurseries, most conifer species have significant innate primary dormancy. Of course lack of domestication in conifer species is understandable; breeding programs are still rather juvenile especially compared to the typical lifecycles of our tree species. And few would argue that reducing dormancy to negligible levels in conifers is a much more arduous task when compared to any current domesticated crop. Fortunately, seed dormancy in species used for reforestation efforts is still a much needed trait; once the window of seed production and seedling establishment associated with the first generation in the nursery has passed, the transition from dormancy to germination of mature dispersed seeds of the next generation must then rely on signals from the 'natural surroundings'. Thus retaining an inherited mechanism for seed survival is an utmost priority. As such, conifers are rather unique among crops that are cultivated - seed dormancy remains a necessary genetic trait for re-establishment of renewable natural stands. Accordingly, rather than attempting to breed out inherent dormancy mechanisms, forest nurseries require efficient dormancy-breaking treatments to ensure timely, economical production of vigorous seedlings.

Seeds of yellow-cedar (formerly known as *Chamaecyparis nootkatensis,* and now referred to as *Callitropis nootkatensis*), true fir (*Abies*) species, and western white pine (*Pinus monticola*) all display deep dormancy at dispersal (***5-7***) and are prime examples of species that require efficient dormancy breakage in tree seed nurseries. Their deep physiological dormancy is, of course, due to the natural habitat in which each species exists.

The natural habitat of yellow-cedar occurs along the Pacific Northwest, through Oregon to British Columbia, and northward to Alaska (***8,9***). Yellow-cedar has a long reproductive cycle, taking between 2 and 3 years to produce seed (***10***). Problems with yellow-cedar seed production often occur because of poor reproductive success in its natural habitat. In seed orchards, which are usually established at lower elevations (***11***), cone production is likewise inconsistent from year-to-year. Non-optimal environmental conditions associated with seed orchards (e.g. low elevation, warmer temperatures, and prevalence of insects) and may further lead to problems with seed maturation, and often seeds are empty or only partially filled at maturity and the rates of seed abortion are high. We have also observed a high frequency of seeds in which there is development of the megagametophyte in the absence of the embryo. Exacerbating problems of poor seed production are problems associated with inadequate dormancy termination; as a result, this species is at a competitive disadvantage. In nature, only a low percentage of seeds will germinate the first year after dispersal. The majority of seeds need longer durations (sometimes up to another year) of moist chilling to break dormancy. Throughout this time, seed numbers can decline dramatically due to consumption by birds and small animals or deterioration caused by fungal attack (***12***). Currently, the common or traditional method of breaking dormancy of yellow-cedar seeds in a lab or forest nursery setting is approximately three months in duration, consisting of a three day running water soak (10-20°C), four weeks of warm 'stratification' (in

which seeds are maintained in the moist state at ~21°C), and eight weeks of moist chilling (2-4°C) (*7,13*).

Western white pine grows along the west coast in southern British Columbia and southward through Washington, Oregon, and northern California. In the interior of North America, western white pine can be found in pockets of Idaho and Montana (*14*). Western white pine was a very important commercial species in the 19th century; however, due to the North American introduction of white pine blister rust from infected pines in Europe and the subsequent death of a great proportion of the trees, its commercial impact is limited even today. However, rust resistant stocks of western white pine are being produced and the impact of the disease today has mostly been mitigated (*15,16*). In order for germination to be elicited, seeds must be subjected to a prolonged moist-chilling treatment for 3 months or longer (*17*). The standard method used in British Columbia (e.g. at the BC Ministry of Forests and Range, Tree Seed Centre, Surrey, BC) is a 14 d running-water soak (10-15°C) and 98 d of moist chilling at 2°C (*13*). Yet, even with this lengthy moist chilling treatment, the germination capacity of different seedlots can be vastly different. Differences in germination performance can also occur when the same seedlot is chilled at different times, or when there are large numbers of seeds subjected to the treatment (e.g. in a commercial operational setting) (*17,18*).

Pacific silver fir (*Abies amabilis*) trees grow throughout Oregon, Washington, and north through coastal British Columbia into southern Alaska. Subalpine fir (*A. lasiocarpa*) has one of the widest distributions of any fir; its habitat occurs from New Mexico, Arizona, and northern California into Oregon, Washington, and north into British Columbia and Alberta, stopping in southern Alaska and the Yukon (*19,20*). Seeds of true fir species, including Pacific silver fir and

subalpine fir display pronounced seed dormancy at maturity. Accordingly, breaking dormancy in these species requires a prolonged moist chilling treatment of 3 to 4 months (***21-23***). In addition, germination of these firs can be impaired by seed pathogens and select seedlots can have high numbers of empty seed (***24***). Seeds of the true firs can be prone to germination during moist chilling; thus, a re-drying of the seeds during chilling has been incorporated to prevent this (***21***). A typical protocol for dormancy termination of Pacific silver and subalpine fir seeds consists of soaking of seeds for 2 days, followed by moist chilling (2-4°C) at >45% moisture content for 4 weeks. Subsequently, seeds are subjected to a "dry-back" (to 30-35% moisture), which is employed to inhibit germination and control fungal growth during moist chilling. Following "dry-back", the seeds are moist-chilled for an additional 8 weeks. Thus the total protocol is approximately 3 months in duration (***13***).

Below we review and summarize modified dormancy-breaking protocols for yellow-cedar, western white pine, and the true firs, Pacific silver fir and subalpine fir, previously published as separate entities (***5,6,25***). In brief, after testing various methods to effectively break the dormancy of yellow-cedar seeds (***25-29***), the most effective uses the anaesthetic 1-propanol, combined with a 3-d warm water soak, a 2-d treatment with the hormone gibberellic acid, followed by 30-60 d of moist chilling. For western white pine, a prolonged warm water soak followed by a 75-d moist chilling period is most proficient (***6***). Finally, moist chilling within a solid matrix of Agro-lig (a commercial formulation of humic acids) or peat moss proves very effective for breaking dormancy of Pacific silver and subalpine fir seeds, respectively (***5***). Compared to traditional dormancy-breaking methods, the modified protocols significantly reduce the time needed to terminate dormancy. The methods are effective for various seedlots within each species, and they further lead to synchronous germination within the seed population. For

yellow-cedar, the modified protocols proved to be further effective in promoting vigorous growth in a forest nursery greenhouse setting and after planting at natural stands. These protocols described below in detail are the collaboration of several years of work in the laboratory of A. Kermode.

2. Materials

2.1 General Items

1. Polystyrene Square Container 156C, 10.95x10.95x3.5cm (Hoffman Manufacturing, Oregon, USA).
2. Seedburo K-22 seed germination paper (Kimpak), No. 87, 25.4 x 35.6 cm (Seedburo Equipment, Illinois, USA) , cut to fit inside container described in 1 above.
3. Whatman 3MM Chromatography Paper, 35 x 45 cm (VWR Canlab, Edmonton, AB, Canada), cut to fit inside container described in 1 above.
4. Forceps (Fine Science Tools, North Vancouver, BC, Canada).
5. Analytical scale (for milligram measurements).
6. Laminar flowhood for sterile work and air drying of seed (ENVIRCO, Sanford, NC, USA).
7. Spoonula or Spatula.
8. Mesh bag, made from vinyl-coated fiberglass screen material available from most building supply stores.
9. Stainless steel tea ball, available from most grocery stores.
10. Double de-ionized sterile water (dH_2O) (double de-ionized-H_2O, dd H_2O, is sterilized by autoclaving).

11. Water bath (approx. 10-12 L) (e.g. Thermo Scientific, Newington, NH, USA)
12. Refrigerator capable of maintaining 2-4°C
13. Controlled temperature growth chamber (e.g. Conviron TC16, Controlled Environments Ltd, Winnipeg, MB, CAN)
14.

2.2 Dormancy Termination of Yellow-Cedar Seeds

1. 1-Propanol (Anachemia Science, Montreal, Canada)
2. Gibberellic Acid A_3 (GA_3) (Sigma-Aldrich, Oakville, ON, CAN)
3. Petri Dishes (10 x1.5 cm)
4. Parafilm
5. Mature seeds of yellow-cedar. In our case, we obtained all seedlots from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada). Seeds should be maintained at -20°C before use. In addition to the 7 seedlots of yellow-cedar (30156, 41409, 07458, 43697, 47467, 42313, and 06731), open-pollinated seed from parent trees 13-6 and 19-8 were obtained from the BC Ministry of Forests and Range, Lake Cowichan Research Station, Lake Cowichan, BC (courtesy of J. Russell).

2.3 Dormancy Termination of Western White Pine Seeds

1. Petri Dishes 10 x 2.5 cm
2. Optional: 70% Ethanol, 30% sterile water
3. Optional: 10% Commercial Bleach, 90% sterile water

4. Optional: Corning 150 mL Tube Top Vacuum Filter System (Fisher Scientific, Ottawa, CAN)
5. Mature seeds of western white pine obtained from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada) including seedlots 08006 and 03727 (courtesy of Dave Kolotelo and Dean Christianson). Seeds should be maintained at -20°C before use.

2.4 Dormancy Termination of Pacific Silver Fir and Subalpine Fir Seeds

1. 30% Hydrogen peroxide (H_2O_2) (EMD Chemicals, Gibbstown, NJ, USA).
2. Agro-Lig Greens Grade (humic acids with particle sizes between 0.212 - 1.29 mm) (America Colloid Company, Reeder, ND, USA).
3. Peat moss (Lakeland Peat Moss Ltd., Edmonton, AB, Canada) sieved with 1.4 mm testing sieve (see 6 below).
4. 150-mL sterile sample bags (Fisher Scientific).
5. Testing Sieve #14, 1.4 mm screen opening (VWR Canlab).
6. Mature seeds of Pacific silver and subalpine fir obtained from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada) including seedlots 24904 and 35573 (courtesy of Chris Monnon, Dean Stewart and Craig Wickland). Seeds should be maintained at -20°C before use.

3. Methods

3.1 Dormancy Termination of Yellow-Cedar Seeds

1. Following removal of the seeds from storage at -20°C, seeds, in batches of 150, are soaked within tea strainers or mesh bags in a 30°C water bath for 3 d.
2. Seeds are then transferred to Petri dishes containing 30 mL of 50 m*M* 1-propanol. The Petri dishes are sealed with Parafilm and gently agitated at 70 rpm on a platform shaker for 24 h.
3. Seeds are transferred to a Petri dish containing 30 mL of 200 mg/L of GA_3. Plates are sealed and agitated as in **Step 2** for 48 h.
4. The seeds are then placed directly on a moist substratum within a polystyrene container for 60 d at 4°C in the dark. The moist substratum consists of 1 layer of Whatman 3MM paper and 1 layer of Kimpak in a square polystyrene container (10.95x10.95x3.5cm) moistened with 30 mL of sterile ddH_2O.
5. Following moist chilling, seeds are divided into replicates of 50 seeds each and placed on a moist substratum in polystyrene containers for germination. This moist substratum is identical to that described in **Step 4** except 50 mL of ddH_2O is added.
6. Seed containers are placed in germination conditions: 30°C days, 20°C nights with an 8-h photoperiod; light intensity at 25 µmoles m^{-2} s^{-1}, PAR 400-700 nm.
7. Germination counts can be monitored daily for the first 15 d followed by once every three days for 15 d (30 total d).

3.2 Dormancy Termination of Western White Pine Seeds

1. Following storage of seeds at -20°C, western white pine seeds are allowed 1-2 h to equilibrate to room temperature. During this time seeds can be counted into needed replicates for subsequent experiments. Alternatively, if dealing with large numbers of seeds, one can calculate the number of seeds per gram and weigh the seeds.

2. Optional: Seeds can be sterilized by soaking in 70% ethanol for 5 min, then 10% commercial bleach for 3 min. This is followed by rinsing with sterile water for two 5-min periods. We often use bottle-top filters to ease in the sterilization of seeds (e.g. Corning 150 mL Tube Top Filter).
3. Seeds are placed into water-penetrable containers such as stainless steel tea balls or, if using larger quantities of seeds, into screen or mesh bags.
4. Seeds are soaked for 10-14 d in running tap water (approx 10-15°C) or in a 25°C water bath with water exchanged daily (*see* **Note 1**). If you are using aseptic technique, the seeds can be soaked in 50-mL conical tubes or Erlenmeyer flasks with gentle shaking.
5. Following the water soak, water is drained and the seeds are placed on Whatman 3MM paper in a flow-hood to dry the surface moisture off of the seeds (*see* **Note 2**).
6. Seeds (<250 per container) are placed on Whatman 3MM paper supported by K-22 germination paper in a square polystyrene container, to which 25 mL of ddH_2O is added. If you are using aseptic technique or have small sample sizes (e.g. 25-30 seeds per replicate), deep dish Petri plates (10x2.5 cm) are used and 12.5 mL of ddH_2O is added.
7. Seeds are placed to moist chill at 2-4°C for 98 d in the dark (*see* **Note 3**).
8. Following moist chilling, the seeds can be transferred to germination conditions. The seeds are transferred to new square polystyrene containers (50 seeds per dish) or deep dish Petri plates (25 seeds per plate), set-up as mentioned in **step 6**, except with 50 and 23 mL of ddH_2O, respectively. Germination conditions of 23°C and a16-h photoperiod, or 25°C days, 15°C nights, and an 8-h photoperiod have been used successfully (*see* **Note 4**). Light intensity for both conditions is kept at approx 60-80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR 400-700 nm.

9. Germination counts can be monitored daily for the first 15 d followed by once every three days for 15 days (30 days total).

3.3 Dormancy Termination of Pacific Silver Fir and Subalpine Fir Seeds

1. Following removal of the seeds from storage at -20°C, seeds are weighed to estimate the seed number needed for a typical experiment.
2. Seeds are soaked in a mesh bag submersed in an aerated-dH_2O running-water bath for 3 d at 20-22°C (*see* **Note 5**).
3. Seeds are sterilized for 30 min in 3% H_2O_2 and rinsed several times with sterilized ddH_2O (*see* **Note 6**).
4. Seeds are dried for approx 5 min in a laminar flow hood until the seed-surface moisture disappears. During this time seeds are divided into the replicates for the experiment (e.g. 4 replicates of 50 seeds each).
5. During the final hours of the seed soak, the matrices for Solid Matrix Priming (SMP) chilling are prepared and placed in 150-mL sample bags. For Pacific silver fir seeds, pre-sieved peat moss is combined with 320% sterile ddH_2O on a w/w basis (*see* **Note 7**) and approx 30 mL of wetted-matrix is placed into a 150-mL sample bag together with 50 seeds from **Step 4**. For subalpine fir seeds, Agro-lig Greens grade is mixed with 60% sterile ddH_2O on a w/w basis and approx 30 mL of wetted-matrix is placed into a 150-mL sample bag together with 50 seeds from **Step 4**.
6. Seeds within the various matrices are then placed at 4°C for 8 weeks of subsequent moist chilling (*see* **Note 8**).

7. Following 8 weeks of SMP chilling, fir seeds are removed from the matrices by rinsing in a sieve (1.4 mm screen) with dH_2O water.
8. Approx 50 seeds are then transferred to a square polystyrene container with Whatman 3MM paper supported by K-22 germination paper and 50 mL of dH_2O water is added.
9. Germination is then tested using 21°C days, 15°C nights, with an 8-h photoperiod; light intensity is at 40 μmoles m^{-2} s^{-1}, PAR 400-700 nm.
10. Germination can be monitored daily or every 3 d for a total period of 30 d. To estimate the 'realistic germinability' of a particular seedlot, the ungerminated seeds are then cut open to determine the proportion of unfilled seeds (e.g. seeds missing a viable embryo due to abortion or poor seed developmental conditions, or attach by a parasite or microorganism such as a fungus).

4. Notes

1. Using a higher temperature soak (i.e. 25°C) will decrease the subsequent moist chilling duration (***6***).
2. This step helps minimize fungal contamination and also aids in dormancy-breakage possibly due to increased air exchange (***5,6***).
3. The moist chilling time for western white pine seeds can be shortened to 75 d if a higher temperature soak is used (**Section 3.2, Step 4**).
4. Germination conditions at 23°C constant temperature elicit more effective germination (i.e. germination occurs over a shorter time frame) but 25°C days, 15° nights may more accurately reflect conditions in a forest seed nursery.

5. In our experience, a 3-d soak is necessary to obtain a stable water content (e.g. 45 % for Pacific silver seeds and 50% for subalpine fir seeds).
6. Sterilized water may not be needed at this point and for subsequent moist chilling since most of the fungal contamination, if present, comes from the seeds themselves.
7. Moisture content of the matrices is calculated on a weight to weight basis (w/w) and is calculated based on: weight of water/dry weight of solid matrix x 100.
8. To determine the optimal period of moist chilling we also considered the potential for germination during the chilling period and tried to minimize this value. Thus, longer periods of SMP moist chilling are beneficial to dormancy-breakage and subsequent germination, but unwanted 'pre-' germination was always increased – sometimes as much as 20%.

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Tables

Table 1. Germination percentages of fir seeds after different SMP-chilling treatments and durations. Modified from ***reference 5*** with kind permission from Springer Science and Business Media.

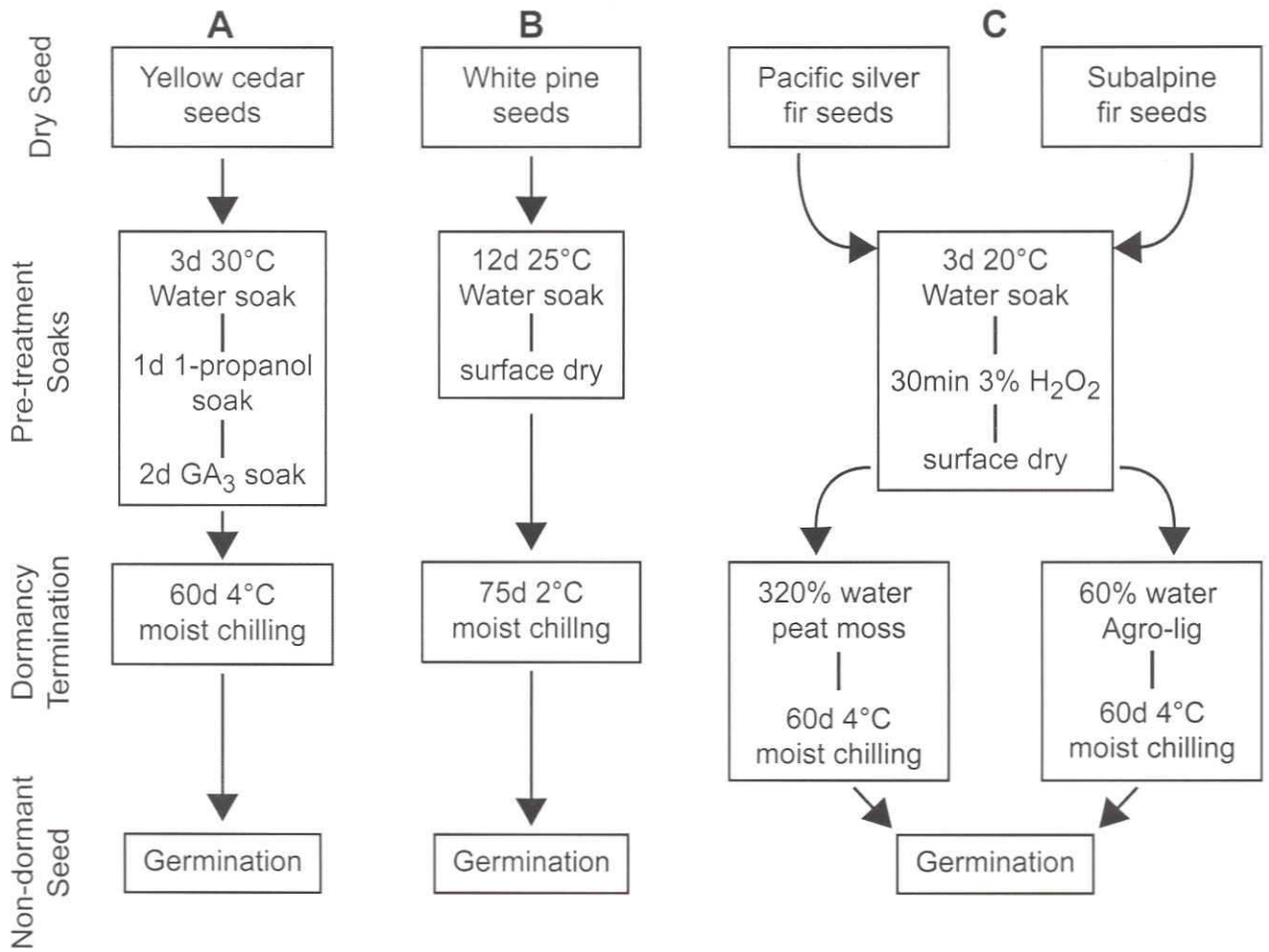
Fir species	Treatment	Moist Chilling Duration 4 wks	8 wks	12 wks
Pacific silver fir	Control: moist chilling	39	44	45
	Agro-Lig Greens Grade	47	64	44
	Sand	61	65	57
	Peat moss	62	**79**	56
	Soaking only	3	3	3
Subalpine fir	Control: moist chilling	41	58	63
	Agro-Lig Greens Grade	63	**83**	70
	Sand	51	71	62
	Peat moss	55	71	68
	Soaking only	12	12	12

Figure Captions

Figure 1. Flowchart summarizing the modified dormancy-breaking methods for yellow cedar, western white pine, and Pacific silver and subalpine firs.

Figure 2. Germination of western white pine seedlots 08006 (A, B) and 03727 (C, D) following a water soak and moist chilling. Seeds were either soaked at 21°C (A, C) or 27°C (B, D) for 10 days, followed by 60, 75, or 96 days of moist chilling. Reprinted from ***reference 6*** with kind permission from the International Seed Testing Association.

Figure 3. Growth of outplanted yellow cedar seedlings two months after transplanting into natural stands near Port McNeill, BC, Canada. These seedlings were subjected to the dormancy-breaking protocol described for yellow cedar (Section 3.1). Reprinted from ***reference 29*** with kind permission from Springer Science and Business Media.


A
B
C
Dry Seed
Yellow cedar seeds
White pine seeds
Pacific silver fir seeds
Subalpine fir seeds
Pre-treatment Soaks
3d 30°C Water soak
1d 1-propanol soak
2d GA3 soak
12d 25°C Water soak
surface dry
3d 20°C Water soak
30min 3% H2O2
surface dry
Dormancy Termination
60d 4°C moist chilling
75d 2°C moist chillng
320% water peat moss
60d 4°C moist chilling
60% water Agro-lig
60d 4°C moist chilling
Non-dormant Seed
Germination
Germination
Germination

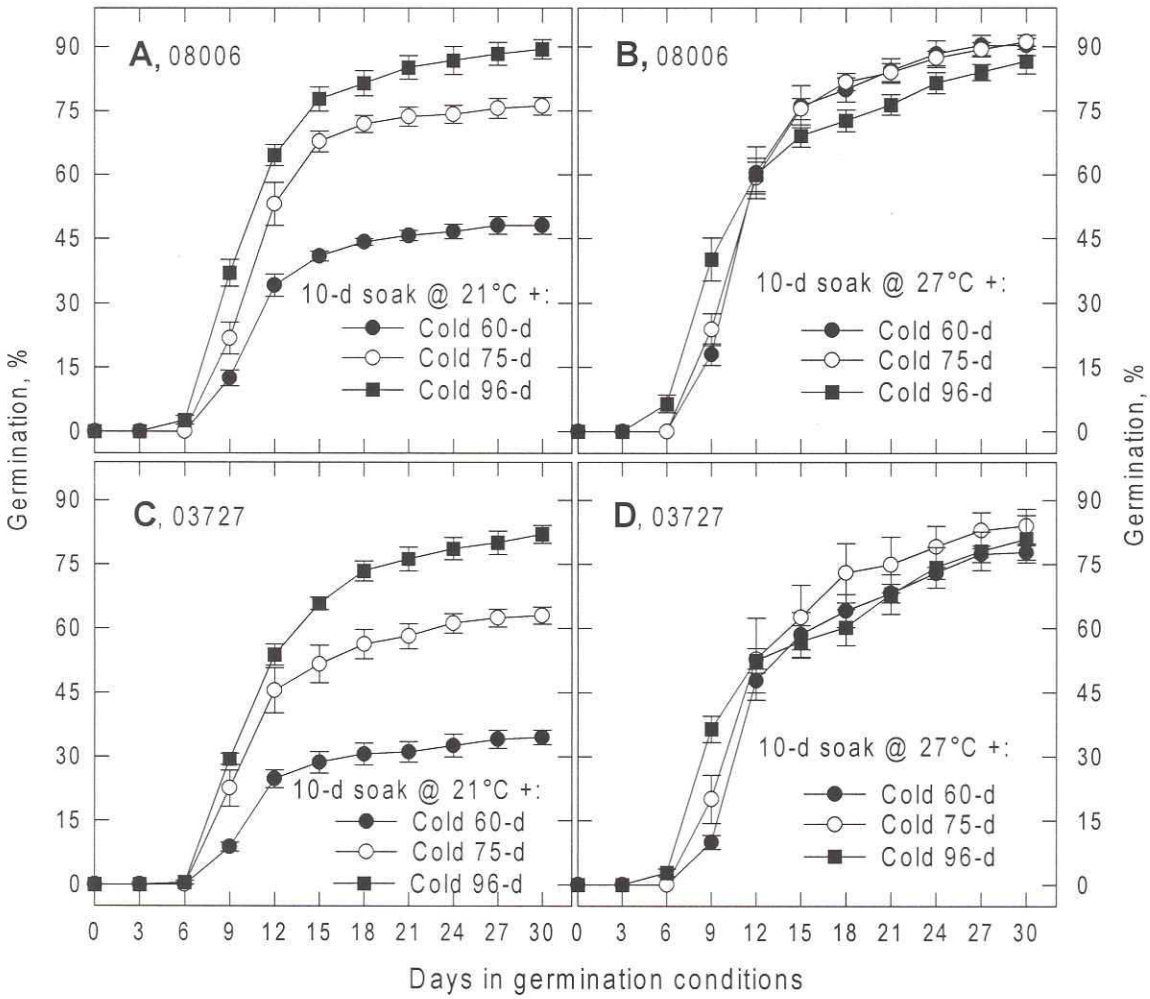

A, 08006
B, 08006
C, 03727
D, 03727
10-d soak @ 21°C +:
10-d soak @ 27°C +:
Cold 60-d
Cold 75-d
Cold 96-d
Germination, %
Days in germination conditions
0
15
30
45
60
75
90
0 3 6 9 12 15 18 21 24 27 30

Figure 4. Photographs of healthy outplanted seedlings two months post-establishment at natural stands. **A.** seedling 94; **B.** seedling 96; **C.** seedling 233. **D.** Cutblock at Western Forest Products research site near Port McNeill, B.C., Canada. Prior to outplanting, the cutblock was flagged. Flags indicated the future yellow-cedar planting sites.