# Coastal Douglas-Fir Controlled-Crossing Guidelines

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## Abstract

Controlled pollination is an essential element in forest tree breeding. Although the concept is simple, the process requires multiple steps, which must be done correctly to obtain successful crosses. As tree-breeding cooperatives of the Pacific Northwest begin a third cycle of breeding and testing, it is timely to describe this process for the major forest conifer species, Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco).

Stimulation by stem girdling and injection of gibberellic acids 4 and 7 is crucial for early flower induction; it can often begin 2 years after grafting into a seed orchard or breeding orchard. Other stimulation techniques (root pruning, calcium nitrate fertilization) can also be effective. Estimates of 10 seeds per female cone and 2 pollen buds to pollinate a female flower can be used for planning. Pollen dried to 6 to 8 percent can be used immediately or stored for future seasons. Important ingredients of successful crossing include attention to detail, constant vigilance, and exact timing, especially when bagging, harvesting pollen buds, and applying pollen to receptive female flowers.

## Introduction

Cooperative tree improvement of Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) has proceeded fully through two cycles[1] and now stands on the cusp of the third cycle. A tree-improvement cycle includes selection of superior trees to use as parents, controlled breeding to produce fully pedigreed families, replicated progeny tests to determine the genetic worth of the parents, and reselection of elite parents and superior progeny to begin the next cycle (Howe and others 2006). As we move into the third cycle, we build on the considerable efforts of past tree breeders. These early forest geneticists often had to make crosses in the tops of selected plus trees in natural stands (figure 1; Ching 1960, Orr-Ewing 1956). Today, all crossing is completed in either established seed orchards or breeding archives (figure 2).

In recent years, much institutional knowledge has been lost as experienced tree breeders have retired or moved on to other pursuits and as important tree-breeding centers have been downsized or closed. Most applied publications date back decades (for example, Ching 1960, Orr-Ewing 1956, Wilson 1969) and, although quite informative, do not include many recent advances or adequate details on a number of steps. Although timber-growing organizations now employ a fraction of the skilled staff their predecessors once did, timelines are aggressive, and the need for efficiency is higher than ever before. At the same time, mating designs call for making between 200 and 400 full-sib crosses within each cooperative third-cycle breeding zone, adding up to several

**Figure 1.** How it once was—crossing on a first-generation parent tree in 1976. (Photo by Marc Vomocil, Starker Forests, 1976)

**Figure 2.** Breeding orchard specifically designed for third-cycle crossing. (Photo by Keith Jayawickrama, Oregon State University, 2012)

[1] First cycle: 1966–2001; second cycle: 1984–present; third cycle: 2008–present.

thousand Douglas-fir crosses across the Pacific Northwest (PNW). A critical need exists for scientists to document successful controlled-crossing techniques for Douglas-fir so that the next generation of tree breeders has the tools necessary to efficiently and cost-effectively complete mating designs.

This article describes the necessary information to conduct a Douglas-fir crossing program. Where more than one way exists to achieve a given goal, each is discussed in turn. Much of this information is gathered from practitioners in the field rather than from published literature, which is limited. A short section on information specific to western hemlock (*Tsuga heterophylla* [Raf.] Sarg.), the secondary conifer species in cooperative PNW tree-improvement programs, is also included.

## Controlled Crossing

Controlled crossing is an important part of tree-improvement programs (White and others 2007). Conditions in a controlled-breeding environment differ from natural stand conditions in at least six key ways:

1. For maximum cost efficiency, breeding cycles are accelerated so that trees are stimulated to flower and produce seed at much earlier ages.
2. Instead of allowing female flowers to be open-pollinated by a mix of abundant natural pollen, pedigrees are controlled to a single male parent, producing full-sib families or mixtures of pollen from known male parents for polymix crosses.
3. Instead of waiting for natural seed production to occur every 7 to 10 years, breeding trees are encouraged to flower every 2 years.
4. Instead of remote, steep, and sometimes high-elevation conditions, trees are established in farm-like, low-elevation, and fairly flat conditions.
5. Grafting is used to establish seed orchards and breeding orchards (Jayawickrama and others 2009). Instead of reproduction by a single-genotype tree developing naturally from seed, a composite tree is built via grafting, using a root system from compatible rootstock, with the reproduction occurring on selected donor tissue known as the scion.
6. Controlled breeding is conducted on trees that ideally can be reached from the ground or from short ladders.

Although the term is taxonomically imprecise, tree breeders commonly use “flower” when referring to floral structures in trees. Before flowering, the term “female bud” or “cone bud” is used for the structure that produces female flowers, and the term “male bud” or “pollen bud” is used for the structure that produces male flowers. Female flowers produce seed cones, and male flowers produce pollen cones. A conelet is a female flower that has been pollinated but is not yet fully mature. We use this accepted terminology throughout the article.

Although Douglas-fir is not the most difficult tree species to breed successfully, it is certainly not the easiest to breed. The reluctance to flower and the abortion of conelets because of frost and other factors are the main obstacles. The time period from pollination to mature seed is relatively short, however; crosses made in April yield mature seed about 5 months later. With diligence, effort, and attention to detail, PNW tree breeders have successfully completed an estimated 25,000 controlled crosses of Douglas-fir during the past 50 years (compiled from estimates by Jeff DeBell, Washington Department of Natural Resources; Michael Stoehr, British Columbia Ministry of Forests; Jim Reno, Weyerhaeuser Company; the Northwest Tree Improvement Cooperative; and the authors’ personal experience).

## Flower Induction

To quickly complete a given mating design, flower induction is required. In most PNW seed orchards, using a flower induction treatment, also referred to as stimulation, has been standard practice for many years, resulting in consistently reliable seed crops. Flower induction treatments applied in the spring take effect that summer, and the resulting pollen buds are observable by fall. Cone buds are less obvious but are usually quite evident by February of year 2. A healthy tree can be stimulated every other year for several years. Stimulating a given tree too often or too aggressively can result in lower vigor, poor seed quality, and, ultimately, death of the tree. In breeding orchards with many ramets (individual trees) per clone, however, it is acceptable to lose some ramets to complete the crossing program faster.

### Gibberellic Acids

Until the early 1990s, tree breeders believed that Douglas-fir orchard trees would not produce appreciable quantities of pollen and seed cones until 10 to 15 years after grafting into a seed orchard. Promising results were obtained by stimulation techniques such as drought stress and root pruning, but such treatments were (and still are) difficult to apply in large orchards. Today, with the ability to apply certain growth hormones, specifically combinations of gibberellic acid (GA), it is relatively simple to get much younger ramets to flower

(Ross and Bower 1989). The type of GA (designated by number), timing, application method, and dosage influence the outcome. Although GA is a powerful tool, it requires careful use to be safe and effective.

### GA4/7

GA4 combined with GA7 (GA4/7) is very effective in stimulating flower production in members of the Pinaceae family, including Douglas-fir, and is available in either crystalline or liquid, ready-to-use formulations. Crystalline GA must be dissolved in ethyl alcohol before use. The liquid form is known by the trade name ProCone™ (Valent Corporation) and is considered the industry standard. As when using all chemicals, the user should consult the label for safe and proper use.

Best results are usually obtained with freshly purchased ProCone. The manufacturer can provide data on how long an individual batch can be used without losing strength. ProCone needs to be stored in a standard refrigerator to prolong its shelf life.

### GA Application

GA application to ramets can usually start 2 to 3 years after grafting (Cherry and others 2007). Tree breeders have different opinions about the best timing for GA application: about the time of vegetative bud break (Cherry and others 2007); when 50 percent of the trees have flushed (Ross and Bower 1991); when most of the trees have flushed (Ross and Bower 1989); and when 50 to 90 percent of the year's vegetative growth has occurred (ProCone label). Anecdotal evidence suggests that younger trees in breeding orchards need to be treated after vegetative bud flush, but older trees in seed orchards respond better to application directly before or at bud break. The ProCone label indicates that, based on application timing, the chemical can stimulate either pollen-bud or cone-bud development.

Although guidelines exist for calculating proper GA dosages, determining the best dosage is as much an art as it is a science. Some clones respond well to lower GA dose amounts, others require higher doses, and yet others can have a phytotoxic response, resulting in excessive needle drop, dieback, and even mortality.

The application rate is based on the cross-sectional area at the point of injection, with the number of holes increasing as tree diameter increases. It is useful to create a spreadsheet ahead of time, measure the diameter of each tree at the point of intended injection and calculate both the amount of GA needed and the number of holes required for that tree. Tree breeders have used a variety of injectors over the years (figure 3). One example is a repeater pipette, which repeatedly delivers the same volume up to 10 times. An injector with the capacity to quickly adjust the dose rate is ideal. In a breeding orchard organized in this fashion, it is possible to treat two trees per minute, using two people. After drilling the downward angled holes into each tree, the product is injected into each hole.

## Stem Girdling

In older orchards, GA may not be needed. Instead, stem girdling is a safer, less expensive, and more predictable stimulation method. In some low-elevation orchards, this technique alone is sufficient to stimulate consistent and reliable flower crops. Stem girdling can be applied when the ramets are at least 5 cm (2 in) in diameter at ground level. Tree breeders have varying recommendations regarding optimal dates for stem girdling: 2 weeks before vegetative bud break, at the time of vegetative bud swell (Ross and Bower 1989, Ross and Bower 1991), 6 weeks before vegetative bud break (Clemo, personal communication 2012), or 1 to 3 weeks before vegetative bud flush (Woods 1989).

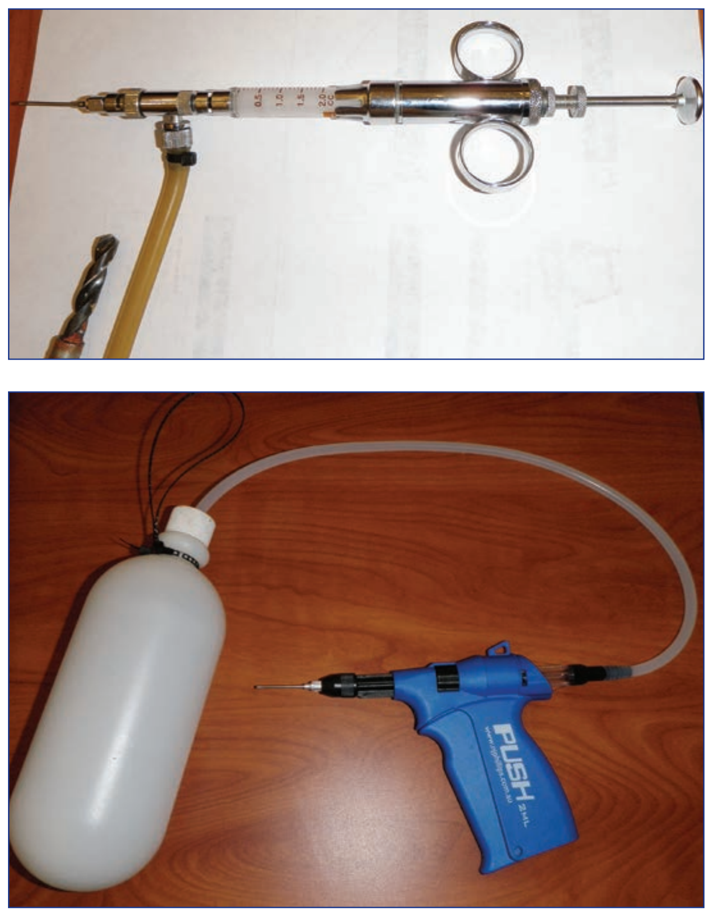



**Figure 3.** Examples of gibberellic acid injectors. (Photos by Keith Jayawickrama, Oregon State University, 2010)

Targeted trees receive overlapping half-circumferential girdles. These trees are cut through the bark and cambium until the saw teeth just reach the xylem (wood), but no deeper (figure 4). Cutting any deeper into the tree results in no benefit and can potentially weaken the stem. Girdling is usually done with a small hand-held pruning saw. Small trees (with a diameter-at-breast-height of 5 to 8 cm [2 to 3 in]) are more safely treated with a knife or a hacksaw. Larger trees are often girdled with a battery-operated, thin-kerf chainsaw.

The first cut needs to be placed at a comfortable working height, with the second cut placed about 1.5 times the stem diameter above or below the first cut, for example, 15 cm (6 in) apart on a 10-cm (4-in) diameter tree. For large trees, space the cuts no more than 30 cm (12 in) apart. Girdles must overlap each other vertically, and the kerf must be left clean of sawdust. New girdles should not be located within an existing scar.

### Calcium Nitrate

Calcium nitrate fertilizer can stimulate flowering in Douglas-fir (Ebell 1972). When applied near the end of March, application rates of 205 to 413 kg nitrogen per ha (200 to 400 lb/ac) have been shown to stimulate increased flowering. If an orchard gets a lot of rain, this fertilizer needs to be applied later in the spring to prevent rapid leaching; in drier orchards without irrigation, it needs to be applied earlier to enable rainfall to dissolve the material and move it down into the soil by the time of active root growth.

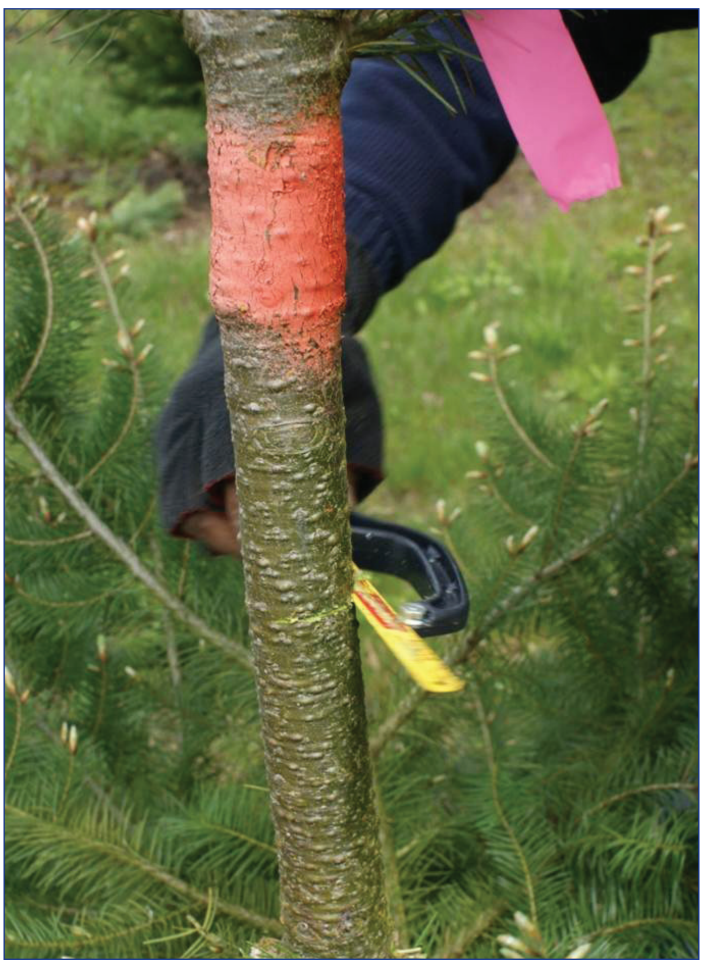


**Figure 4.** Stem girdling on a small ramet in a breeding orchard. (Photo by Larry Miller, Oregon Department of Forestry, 2012)

### Other Stimulation Methods

Girdling, calcium nitrate, and GA may be applied singly or in combination to increase effectiveness. Root pruning (using a tree spade or a single ripper preceded by a rolling coulter) is another option. This process is usually implemented around the time of pollen-bud swell and is most effective when used in conjunction with GA. This treatment is very effective in potted orchards, which may be one reason to establish some ramets in pots. This technique may be too severe to be used every other year.

## Initial Planning Before Crossing Season

Although third-cycle breeding programs need to begin with a clear crossing plan, considerable variation in fecundity and phenology is to be expected, which may necessitate changes in plans both within and between years. Douglas-fir is monoecious, so a clone may be used as a male parent, a female parent, or both. Because most third-cycle material consists of recently grafted ramets—often from juvenile forward selections from second-cycle tests—pollen buds may be limited. A simple database helps to organize the data needed for each clone. Information to be tracked includes the following items:

- Clone number.
- Number of ramets.
- Orchard row-column coordinates.
- Tree size.
- Vigor rating (excellent, fair, or poor).
- Female flower rating (5 = heavy, 0 = none).
- Male flower rating (5 = heavy, 0 = none).
- Female flower maturity (1 = dormant, 5 = post receptive).
- Pollen flower maturity (1 = totally closed, 5 = shed).
- Phenology (early, mid-, or late season).
- Comments.
- Pollen available from previous years.
- Estimated number of existing seeds for a given cross.

Collecting and tracking such data ensures that early trees are bagged or collected first, and late trees are delayed to the latter part of the breeding season.

A good pollen tree has plenty of pollen buds, matures early, is in excellent health, and can be easily worked, either from the ground, a small orchard ladder, or the bed of a pickup truck. An optimal tree for crossing has similar traits but produces more female flowers than male flowers. Considering the relatively short breeding season, ready accessibility of reproductive buds is important.

## Pollen

All stages of pollen collection, processing, storage, and viability testing are comprehensively described in Webber and Painter (1996). Only the highlights, and local experience in Oregon and Washington, will be presented here.

### Pollen Planning

Pollen is rarely limiting in older, first- and second-cycle seed orchards. A good orchard-sized tree can produce more than 100,000 pollen buds per year, enough for several liters of pollen. The challenges are to find such good pollen trees, collect and process the pollen in time for use on bagged female flowers, and keep this pollen viable.

In third-cycle crossing programs, however, pollen is likely to be limited. Experience shows that 1 ml of fresh pollen can treat 10 flowers once. If pollen is limited, however, up to 30 flowers may be lightly treated with this same 1 ml of pollen. Rare pollen lots can also be diluted with either dead pollen or talc, using a maximum ratio (volume-to-volume) of 1:1. Because seed yields may be reduced, pollen “stretching” needs to be reserved for only extreme situations. Pollen lots need to be labeled as “use sparingly” or “use liberally.”

Another way to calculate pollen needs is to assume two pollen buds per female flower. A target of 200 seed for a cross would therefore require 10 cones and at least 40 pollen buds.

Breeding plans and crop survey data need to be compared to determine how much pollen is needed from which clones. When in doubt, err on the side of collecting too much pollen. In some trees, the amount of time required to pick and extract 400 male buds can be nearly the same as for 40 buds. Breeding is considerably faster and seed yields much higher if extra pollen is available. Extra pollen can be stored for use in subsequent years.

When starting a new crossing program, it is advisable to build a stock of pollen from a number of parents before putting much effort into bagging and crossing. Without experienced workers, it is challenging to collect and process pollen in time to pollinate with it during the same year. Flowering involves a 2-year cycle, so a missed opportunity today will not be available again on the same trees for 2 more years.

### Pollen Collection and Forcing

Especially in young breeding orchards, pollen availability is often limiting. Thus, it is advisable to build up pollen inventories for 1 to 2 years before the onset of controlled crossing. The first step is to carefully track the maturation of pollen buds, using a 1 through 5 rating system (Webber and Painter 1996):

1. Dormant.
2. Buds beginning to swell but still closed tightly.
3. Pollen cone breaks through bud scales.
4. Pollen cone elongated, with visible spaces between microsporangia.
5. Pollen cones completely shed.

Using this rating system ensures that pollen cones of most lots will be picked at the optimum time, giving good yields of high viability pollen. The best time to harvest pollen is when pollen cones are at Stage 4, elongated, and resembling a clump of grapes (figure 5). Squeezing a mature pollen cone

**Figure 5.** Mature pollen cone of coastal Douglas-fir that is ready for picking, directly before pollen shed. (Photo by Bill Marshall, Cascade Timber Consulting, 2011)

yields very little liquid; what liquid exists is expected to be yellowish and thick. Those that release clear liquid are not ready to pick. Differences in pollen cone color are normal and bear no relationship to maturation—mature flowers may be yellow, pink, purple, or red.

Early pollen collection combined with forcing (see the description that follows) also is possible, but this collection usually reduces yield and viability. Conversely, if picked too late, pollen buds will be partially shed, also reducing yields.

When days are warm, sunny, and dry, pollen cones can develop from Stage 3 to Stage 5 in a few hours. Even if pollen cones have started to shed, usable amounts of pollen can be acquired by increasing the number of cones collected. Another option is to assess other ramets of the same clone, which may mature later if located in cooler and shadier parts of the orchard. Because pollen cones mature at different rates depending on exposure, it is also important to examine bud development all around the tree. If the pollen crop is large, collecting beyond the optimal window will still give an adequate yield. When the pollen crop is small, however, timing is critical and repeated visits to the same tree to pick at the optimum maturation stage will maximize pollen yields.

Pollen can be transferred from tree to tree on hands, clothing, and tools, which can cause cross contamination of different pollen lots and, thus, loss of pedigree control. Fortunately, sanitation is a simple process. Using rubbing alcohol, spray down skin and tools before beginning work on any tree. If hand cream does not adequately counteract the drying effect of the alcohol, surgical gloves can help protect sensitive skin.

Mature pollen cones need to be collected in lightweight, paper "lunch sacks" labeled with the clone number and date. Alternatively, branch tips with pollen cones may be clipped and placed into larger sacks. Pack each bag loosely, fold over the top, and staple to close. Collected pollen cones need to be stored in the shade or in a cooler during a warm day until transferred to the processing facility. Although undesirable, pollen cones may be harvested when working in the rain. This harvest needs to be done only when the pollen will be processed in a forced-air system to avoid molding. After pollen has been collected from a ramet, the ramet needs to be checked off the field sheet.

Orr-Ewing (1956) and Ching (1960) reported on a system to force pollen indoors, which is currently used in one Douglas-fir breeding program in Germany to acquire pollen for use within the same year. They place the end of each clone's freshly cut branch in a glass container with AKN-solution (Heisel 1983) and keep the room at a fairly uniform temperature not exceeding 27 °C (81 °F) during the day, and slightly cooler at night. If branches are cut early and pollen has yet to ripen, the room needs to be maintained at 80- to 100-percent relative humidity (RH); if branches are cut directly before shedding, then the room needs to be kept at 60- to 70-percent RH. Shortly before shedding, the area below the branches needs to be cleaned and lined with newspaper. Pollen sheds over a 2- to 4-day period, and can be collected each day. Pollen shed can be forced by slight tapping of the branches.

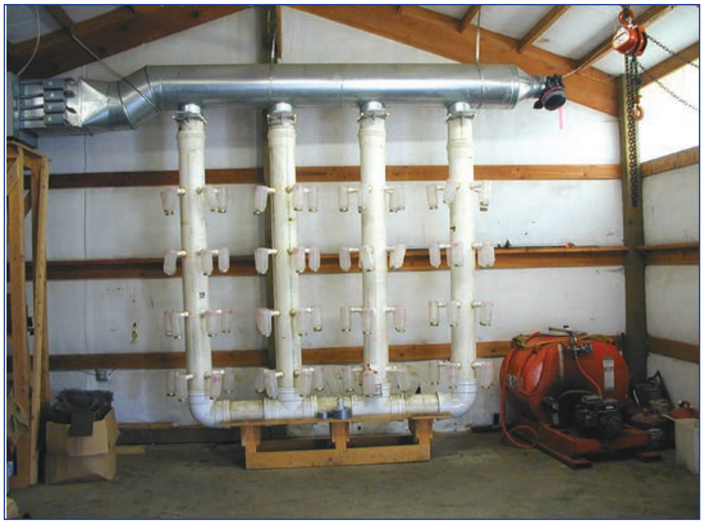
## Pollen Processing

Pollen must be dried to a moisture content (MC) of 6 to 8 percent to be used efficiently and stored properly (short- or long-term). Thus, having a warm (at least 27 °C [81 °F]), dry room enhances pollen cone handling and processing results. Because it is frequently rainy during the pollen collection season, a clothesline needs to be installed on which bags of freshly collected pollen cones may be hung for initial drying. The best system for pollen processing is a forced-air apparatus that uses screened funnels with attached containers to collect shedding pollen. Examples of good pollen-processing facilities are described in Franklin (1981) and Webber and Painter (1996) references and shown in figure 6. Pollen can be processed under less optimal conditions, but will require more time and return more variable results.

Using a forced-air system, pollen cones collected at Stage 4 will shed most of their pollen in 24 to 36 hours. Tapping the funnels occasionally helps pollen fall into the collection vial; this pollen will be clean and free of debris after passing through the screen. By 48 hours, forced-air-dried pollen will reach the target MC and can either be used immediately or stored in a freezer. The MCs of a few early collections need to be tested before freezing to ensure that the target MC has been reached. Pollen dried to the proper MC will, when swirled in the vial, flow like water.

Without a forced-air system, good supplies of high quality pollen can still be collected, but the process will take longer, the MC will be more variable and therefore the pollen will not be reliable after long-term storage. Under this scenario, pollen needs to be sieved after collection to remove pieces of bud scales, insect parts, and other nonpollen debris, and then bottled for immediate use or refrigerated for short-term storage.

Best results are obtained when collected pollen cones are processed immediately. If the cones cannot be processed immediately, the paper bag containing buds can be placed in a refrigerator at about 3 °C (38 °F) for up to 10 days (Webber and Painter 1996).



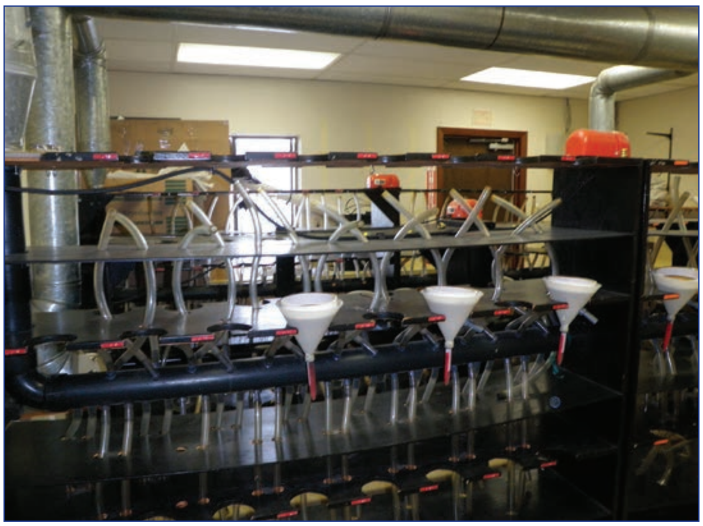


**Figure 6.** Examples of pollen extraction systems. (Photos by Keith Jayawickrama, Oregon State University, 2012)

## Pollen Storage

Unused pollen collections from the same clone can be combined at the end of the season and placed in storage. Pollen needs to be resieved as needed and checked for MC. Before long-term freezer storage, pollen lots must be dried to 6 to 8 percent MC to avoid damage. Depending on the measuring device used, varying amounts of pollen are needed to accurately determine MC. Contact the authors for examples of equipment used to measure pollen MC.

Regardless of the type of storage container (glass, plastic, or aluminum-foil pouches), it is very important to seal the container well. Sealing the lids with Parafilm™ provides an extra layer of protection against air infiltration. Label all pollen containers and lids clearly. Pollen can be stored in graduated bottles, which makes it easier to estimate the volume. Storage containers need to be filled completely to remove as much air as possible before storage.

Storage in vacuum-sealed containers or those filled with an inert gas such as nitrogen, maintains pollen viability longer than does storage in containers containing ambient air. Another approach is to use an array of container sizes so that most will be completely filled. Under such conditions and when frozen at -18 °C (0 °F) in a standard household freezer where no temperature fluctuations exists, pollen viability will usually remain high for 2 to 3 years. Storage for longer periods of time requires more careful preparation and much colder storage temperatures.

## Pollen Inventories

Existing pollen lots need to be tested by February of the year of intended use. Germination testing procedures for Douglas-fir, as described in Webber and Painter (1996), are relatively straightforward and have been widely used in the PNW. Pollen lots with at least a 50-percent germination rate are suitable for use (Webber and Bonnet-Masimbert 1993). Pollen with lower viability may be used, but reduced seed set is likely.

# Controlled Pollination

## Female Flower Development

Webber and Painter (1996) identify five developmental stages of Douglas-fir female buds and cones:

1. Dormant.
2. Beginning to elongate.
3. Approaching bud burst.
4. Receptive flower with 30 to 40 percent of the bracts exposed.
5. Post-receptive conelet.

It is very important to follow flower development closely so that pollination bags are installed at the proper time for successful controlled crossing.

Maturation of female flowers depends on weather and crown position, with as much as 4- to 8-days difference in optimum receptivity in different parts of the tree (Webber and Painter 1996). Flower maturity varies even within the same pollination bag. Until the tree breeder becomes experienced, it is necessary to assess the receptivity of each bag on numerous occasions throughout the spring. As with pollen, flower color is not an indicator of its receptivity.

Douglas-fir cones usually have 35 scales, each with two ovules, for a maximum of 70 viable seeds per cone. Ovules at the basal and at the distal portions of each cone usually are not fertile, so the middle two-thirds of each cone produce most of the viable seed. For predicting breeding workloads, assume a recovery of 10 filled seeds per pollinated cone. Individual flowers stay receptive for 6 to 8 days after bud burst, with the optimal time to pollinate being days 2 through 8 (Webber and Painter 1996).

## Flower Inventories

In most of the PNW, male and female buds develop sufficiently by late February to be readily identifiable (figure 7). At this point, we can determine which clones will mostly be used for pollen production, for females, or for both based on the relative abundance of male and female buds. The sooner trees are identified for use as females (such as the tree in figure 8), the more efficient breeding will be for that year because of the time required to install pollination bags.

**Figure 7.** Well-developed male (top) and female (bottom) buds. (Photos by Larry Miller and Lisa Clemo, Oregon Department of Forestry, 2012 and 2013)

## Pollination Bags

Pollination bags are used to isolate female flowers from nontarget pollen to ensure complete control of the pedigree. Ideal pollination bags:

- Exclude 100 percent of nontarget pollen.
- Are strong enough to handle wind gusts, abrasion from adjacent branches, rain, snow load, and sun.
- Reduce heat transfer.
- Permit exchange of air and water vapor.
- Include a window to allow cone development to be checked without removing the bag.

Bags with all the above characteristics are relatively expensive; however, the temptation to cut costs on bag quality needs to be resisted. It is very frustrating and costly to have most of the bags torn after a windstorm or heavy rain. Such

**Figure 8.** Prolific flowering on a very small ramet. A major challenge facing the breeding program is making such flowering the norm rather than the exception. (Photo by Keith Jayawickrama, Oregon State University, 2012)

a setback can delay the crossing program by 1 year or more. It is more cost-effective to buy a roll of bagging material and cut the bags to fit different-sized branches. Sources of pollination bags are somewhat limited; contact the authors for known suppliers.

**Timing and Placement of Pollination Bags**

The best time to begin installing pollination bags depends on tree phenology and the size and scope of the breeding program for a given year. In most years, bagging is typically done in late March, before bud burst and pollen flight. When a large breeding effort is planned, early bagging may be warranted, although this approach can subject the bags to longer periods of potential damage from wet, windy weather. Waiting too long to install bags increases the risk that female flowers will be contaminated by unknown pollen (by the time the female flowers are receptive as in figure 9, it is too late).

To account for variations in fertility and losses from frost, we often use a factor of 10 for Douglas-fir. For example, to achieve a target of 200 seeds, 20 female flowers need to be bagged. Barring a catastrophe, applying this factor is usually sufficient. Crossing results are notoriously variable among clones, however. After we know how specific clones produce, we can adjust the factor accordingly in subsequent years.

Third-cycle crossing will involve multiple bags on small ramets (figure 10). From the standpoint of risk management, hanging all bags on one tree is not a good practice—a total loss could be incurred because of localized frost, a ramet could snap off in a windstorm or when loaded by snow, as examples. Where practical, it is best to distribute pollination bags across at least two ramets of a clone. Maintaining correct identity of crosses is the top priority, and any strategy for using the same tree for multiple crosses must be balanced against the risk of confusing cross identity.

**Figure 9.** Female flower of coastal Douglas-fir at time of peak receptivity. (Photo by Larry Miller, Oregon Department of Forestry, 2012)

Bags need to be placed in the easiest portion of the crown in which to work. An optimal area will be easily accessible and will have many females and few males. The target number of flowers to be bagged on a given tree can be spread across several bags, each enclosing 6 to 8 flowers. This allows for some flower abortion due to injury from cold and from rubbing against the bag. Experience shows that fewer than 4 flowers per bag significantly increases labor costs and more than 10 flowers per bag causes crowding, which often leads to cone abortion unless large bags are used. Bags need to be placed in the same general area, yet not so close together that they may rub against each other in the wind. Placing the bags in a similar orientation promotes even flower development, thereby reducing the number of pollination visits.

**Installation of Pollination Bags**

A sturdy wire hook attached to a pole is helpful for pulling branches to within working distance. Lifts and ladders may be needed when crossing tall trees. Ladder use has a surprisingly high accident rate, so safety training is strongly advised, with careful attention to applicable regulations.

Pollen buds inside the bag or directly outside it's opening must be removed before female flowers are bagged. Watch for

**Figure 10.** Multiple pollination bags on a small ramet. Ramets of this size will be the norm as cooperative third-cycle crossing gets under way. (Photo by Keith Jayawickrama, Oregon State University, 2012)

small hidden pollen buds, especially between and underneath branches. Vegetative buds need to be removed from branch tips, otherwise the expanding shoots will elongate inside the bag, causing overcrowding. Do not leave sharp twigs in or near the bag because they can tear holes in the bag. Trim off excessive foliage inside the bag to reduce crowding. Because pollen is often applied by opening the top of the bag, place bags so the enclosed flowers are easily visible and reachable from the top of the bag.

Pollination bags act like parachutes in the wind, so small branches may break if not adequately reinforced; splints made from twigs or bamboo work well for this purpose. Internal supports made from aluminum wire can be formed into "halo" or spiral shapes and then tied to the flowering branch to help keep the bag "inflated." Do not let flowers touch the inside of the bags—this can cause a high rate of flower loss. Bags placed on lateral branches can be supported by tying the branches to the main stem. Additional support is generally required on smaller ramets or on those with small diameter branches.

Dacron batting works well as a pollen gasket applied around the branch where the pollination bag will be attached. This gasket needs to be located close enough to the branch ends to allow about 5.0 cm (2.0 in) of bag extending below the Dacron for an adequate seal and about 5.0 to 7.5 cm (2.0 to 3.0 in) of extra room between the branch tips and the top of the bag so the bag can be opened and closed several times.

Write the number of flowers contained in the pollination bag on its outside with a permanent marker. Next, open the bag, fully extending all the gussets, and slip it over the branch. To hold the bag in place, apply a zip-tie, over the area padded with Dacron batting. Turn the bag's window away from the sun, and tighten the zip-tie. Finally, trim away external foliage that might damage the bag.

Pollination bags cause a greenhouse effect, thereby accelerating maturation of the enclosed flowers. If labor is short and work must begin relatively early in the season, flag and prepare the intended branches as described above, but leave the bags off. Prepared branches can then be quickly bagged directly before the breeding season.

Check each bag periodically, especially after a heavy windstorm. If a bag was intact before the storm and is assessed for damage immediately afterward, it is reasonable to assume minimal pollen contamination under wet/windy conditions. Minor pin-holes can be repaired by stapling a crease over the damaged portion of the bag or sealing with duct tape. If bags must be replaced, transfer all labeling and notes to the replacement bag. If the exclusion of foreign pollen cannot be assured, remove the bag and delete the cross, updating the record accordingly. If rainwater accumulates in a bag, open it and let the water out to prevent molding. To minimize this problem, avoid bagging limbs that are oriented horizontally or downward.

## Pollen Application

Perfect control pollination conditions are dry foliage, calm winds, and minimum condensation inside the pollination bag. Phenology assessments, pollen preparation, and other tasks are therefore best done in the morning, with pollination occurring from mid-day into the evening. If female flowers and pollen are scarce, complete as many crosses as possible in a given year, and finish the rest in a later year. If pollen is insufficient to complete even one cross, it is better to wait until a later year when more pollen is available.

Each day, pollen lots intended for use need to be transferred into syringes or bottles in the lab using funnels. Pollen needs to be taken to the field in coolers. When tree breeders are in the field, any pollen that is not in use needs to be stored in a cooler, keeping the cooler in the shade as much as possible. Many methods are used to apply pollen to female flowers, including using spray bottles, syringes, and brushes, and those tools are described in the following section.

A highlighted orchard map is useful for planning and tracking each day's workload. Before pollinating the intended tree, verify that it is the correct clone, and sanitize your hands and forearms with rubbing alcohol. Next, carefully inspect each bag. If any pin-holes or abrasions exist, repair, replace, or remove as appropriate. If any unshed pollen flowers exist, remove them. If missed pollen cones are at or near pollen shed, remove the bag and delete the cross.

For all completed crosses, use a ball-point pen to label a write-on aluminum tag for each bag. Such tags need to be marked with a sequential bag number (circled), and the pedigree (Female # x Male #) of the cross. Securely fasten each tag to the appropriate branch directly below the base of the corresponding pollination bag. Before leaving for the next tree, update the field notes and double check to ensure that all bags have been checked or pollinated, all bags have been resealed and are free of damage, all records are complete and legible, and all pollens have been returned to the cooler.

### Spray Bottle and Syringe Pollination

If pollen is abundant, it can be poured from a test tube or squeezed from a plastic bottle onto bagged flowers. Plastic nose-spray bottles can be used as a low-tech, inexpensive option for blowing the pollen onto bagged flowers.

When pollen supply is short, syringe pollination is recommended. Adding a little talc (less than 10 percent) to each pollen lot helps prevent needle clogging. Using large-diameter needles, called "blunts," and by avoiding pollinating when bags are wet can also minimize clogging. Take care to apply pollen directly onto all bracts of each flower. Syringe pollination requires only small needle-holes in the pollination bag, taking care to seal with duct tape when finished.

When using either a syringe or spray bottle, poke a hole in the bag, and blow the pollen onto the flowers (figure 11). Lightly tap the bag to keep the pollen suspended for a few extra seconds. This step will improve the chances of the pollen grains falling onto a receptive flower, thereby improving seed set. A nail tapped into a block of wood may be used to poke a hole in the bag. Seal the hole using duct tape before moving on to the next bag.

**Brush Pollination**

Brush pollination is another good method (figure 12). Camel or horse-hair brushes work well, as do those made from sable (expensive) or squirrel (preferred). Some breeders opt to not use brushes made of synthetic materials, because the pollen tends to cling to them via static electricity. Others use very inexpensive brushes and discard after one use to minimize the risk of contamination.

**Figure 11.** Pollination of Douglas-fir flowers with a squeeze bottle. (Photo by Keith Jayawickrama, Oregon State University, 2012)

If using brushes, have two pollen vials for each male parent clone: one partially filled vial prepared with a brush and one as a back-up vial of extra pollen. If the brush vial is dropped or the pollen becomes damp, transfer the brush to the back-up vial.

For brush pollination, take the pollen from the cooler and carefully open the end of the pollination bag. Remove the brush from the pollen vial and apply pollen to each flower enclosed in the bag. Most tree breeders prefer not to touch the flowers with the brush and try not to allow flowers to rub against the side of the bag. It is good practice for the breeder to place his or her thumb over the top of the vial whenever the brush/cap is off. This practice helps avoid inadvertent pollen spillage, keeps the wind from sucking out the pollen via a Venturi effect, and minimizes the risk of contamination by foreign pollen.

When pollen is in very short supply, the breeder should wait to pick the pollen buds until they have started to shed pollen directly into a small poly bag or vial. Apply this naturally shed pollen from the bag or vial to the female flowers with a small brush. Maturation of the pollen and cone buds must be monitored very carefully to take full advantage of such small pollen crops.

Seal each pollination bag immediately after brush pollination by double-folding the opened end and stapling it shut (three staples are usually adequate).

**Figure 12.** Brush pollination of Douglas-fir flowers. (Photo by Dan Cress, Regenetics Forest Genetics Consulting, 2006)

### Second Pollinations

If pollen is not limited, it needs to be applied generously to each flower to increase seed set. Another option is to pollinate each flower twice. The first pollination needs to occur when roughly 25 percent of the bracts have extended from the bud scales—about a week after the bract tips first emerge. The second pollination needs to occur when about 75 percent of the bracts have emerged.

Before any second pollinations, review the phenology data and breeding records to locate bags that may be ready for repollinating. Check that each bag is still free of abrasions and pin-holes. Repair or replace bags as needed, or delete crosses as appropriate. When bags are replaced, transfer all notes from the old bag to the new. Inspect the enclosed flowers and take detailed notes on phenology. If three-fourths of the bracts are exposed, the flowers can be pollinated again. This repollination often occurs about 3 days after the first pollination, sooner in warm weather, or later in cold, wet conditions.

For bags ready for a second pollination, check that the information on the aluminum tag matches all other records. Use a staple puller to reopen the bag, and carefully repollinate all parts of each flower. Seal each bag when finished.

In years with a long, cold spring, three pollinations may be needed. Careful field notes will identify any bags that still contain immature females at the second pollination.

## Protection and Maintenance After Controlled Pollination

Cone and seed insects occur commonly in seed and breeding orchards. Most damage results from one or more of the following:

- Douglas-fir cone gall midge *(Contarinia oregonensis* Foote).
- Western conifer seed bug (*Leptoglossus occidentalis* Heidemann).
- Douglas-fir cone moth (*Barbara colfaxiana* Kearfott).
- Fir cone worm (*Dioryctria abietivorella* Groté).
- Douglas-fir seed chalcid (*Megastigmus spermotrophus* Wachtl).

Insecticides are available for treating these pests; however, they must be used carefully. On rare occasions, some insecticides can cause pollinated female flowers to abort. Consult the pesticide label for proper application to control cone and seed insects.

After the breeding season, three options exist regarding the pollination bags. Some choose to replace pollination bags with mesh insect bags, such as fiberglass window screening sown into a simple flat bag. Using this type of bag reduces the risk of the cones overheating in the summer sun (compared with the pollination bags), increases the chance of reusing the pollination bags, and keeps insects out. Some breeders choose to leave the original pollination bag, taking care to have the window facing down. Either the mesh or pollination bags will provide a buffer against late cone harvesting because any seeds that shed will remain within the bag; they also make it obvious which cones are products of controlled pollination. The third option is to remove the pollination bag after risk of common cone and seed pests is minimal. If using this option, it is helpful to paint the branch below the pollinated cones or otherwise mark it for ease of future location.

## Cone Harvesting

Harvesting the control-pollinated cones is one the simpler parts of the process, yet improper timing or poor record keeping can waste an entire year's breeding effort. Optimal timing to obtain fully ripened seed varies by orchard and year, but typically occurs in late August to early September. Seed wing color is a much better indicator of seed maturity than cone color. A mature cone has brown seed wings, is somewhat flexible in a lengthwise direction, and floats in water. The greenhouse effect of pollination bags left intact may accelerate cone ripening, so the condition of nonbagged cones on the same trees may not be a good indicator of bagged-cone ripeness. As cones ripen, they dry out and flare open. Flared cones begin to shed their seeds; therefore, unbagged cones need to be harvested directly before they flare since the riper the cones the better the resulting seed.

Some breeders prefer to collect cones from controlled crosses in advance of operational harvests to avoid possible damage or loss of bagged cones. Others breeders prefer to wait until after the operational harvest to assure ripeness, because bags are fairly easy to avoid. If a branch with a bag is broken off, the cones from that bag can be harvested earlier than planned.

After a tree is ready for harvesting, insect or pollination bags are removed if present, and the cones are carefully picked and placed into small sacks with a tight, breathable mesh, such as cloth rice sacks. If cone sacks are large enough and cone collection personnel are fully experienced, multiple bags of the identical cross may be combined. Capture any seeds that have shed into the bag, if bags are present. The cross identity is then transferred from the aluminum tag(s) to a paper tag, the aluminum tag(s) are placed in the sack with the cones, and the paper tag is securely attached to the outside of the cone sack.

If the cones are inside pollination or mesh bags, a second option is to allow flaring to take place, and harvest the entire bag, limb, and cones. The seed and flared cones can then be processed indoors.

Cone sacks must be only one-half full or less; over-filling will interfere with proper after-ripening and cone opening. Cone sacks must be kept dry and off the ground, with good air circulation. For best results, spread sacks in a single layer on wooden racks erected in an open-air shed. Multiple bags of the same cross, and their reciprocals, need to be carefully combined before shipping to the seed plant. Avoid harvesting cones during rainy weather to prevent molding, reduced seed recovery, and reduced seed viability.

Germination tests have shown that seed yield from controlled cross bags can be similar to that of open-pollinated cones, but it is also recognized that germination rates of different crosses can be different. The importance of scrupulous record keeping cannot be over-emphasized.

## Crossing of Western Hemlock

In general, it is easier and faster to reach a target quantity of control-pollinated seed for western hemlock than it is for Douglas-fir. Techniques and protocols for controlled crossing of western hemlock are similar to those used for Douglas-fir, but key differences exist (Webber 2000, authors' personal experience):

- Western hemlock cones are much smaller than Douglas-fir cones, so it is possible to fit dozens of female cones into a pollination bag.
- Female flowers rarely abort by coming into contact with the pollination bag.
- Hemlock cones yield about 15 filled seed per cone, so a single bag can produce a very large number of seed.
- Female flowers have a longer receptive period (up to 12 days).
- Removing pollen buds from pollination bags is time consuming because they are very small and far more numerous.
- Wire or wooden splints are often needed to strengthen the very flexible limbs, but wire internal bag supports are unnecessary.
- The optimum timing for flower induction in western hemlock in general is later than in Douglas-fir.
- Western hemlock cones mature later in the fall, from mid-September to early October.

## Conclusion

The first substantial cooperative third-cycle crossing effort was in the spring of 2012, with about 100 crosses attempted. We expect 100 to 200 crosses to be made each year for the next decade. The plan is to test the resulting third-cycle seedlings and establish new third-cycle orchards starting around 2025. Those orchards are expected to affect the plantations growing through a substantial part of the 21st century: assuming that 40 million trees are planted per year over 15 years, the result could be 600 million trees or about 1.5 million ac derived from third-cycle crossing. It is our hope that these guidelines help make that crossing effort as successful as it can be.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