The Longevity and Fertility of Freeze-Dried Douglas-Fir Pollen[1])

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Introduction

With attention being focused on the science of forest genetics, breeders have become acutely aware of the need for maintaining reserve supplies 02 viable pollen. In addition to eliminating the barrier of time in breeding programs, successful storage of pollen can save time, effort, and money spent in annually procuring supplies of pollen. A study of preservation is requisite to a clear understanding of the physiology of pollen as it relates to the aging process and of the influence of moisture and temperature on longevity of pollen. The objective of the present study was to find a means by which pollen viability in Douglas-fir *(Pseudotsuga menziesii* [MIRB.] FRANCO) could be prolonged.

Research on longevity of pollen has shown that, under proper conditions, pollen is still capable of effecting fertilization after storage for several years. Studies such as those of JOHNSON (1943), DUFFIELD (1954), SNYDER (1961), and KING (1965) have shown that there are wide differences in longevity for each species varies directly with the ambient moisture content and temperature of air to which the pollen is subjected. DUFFIELD and CALLAHAM (1959) reported that viability of pine pollen did not change appreciably when the pollen was stored at -23^0 C for almost a year. Fresh pollen and pollen stored for 10 months appeared equally capable of fertilizing. Such results were obtained with conventional methods of storage, that is, with environments controlled by low temperatures and chemically regulated humidity.

PFEIFFER (1955) was among the first to study the effect of lyophilization, or freeze-drying, on viability and longevity of pollen. She concluded that, with the technique followed. lyophilization did not improve the keeping qualities of lily pollen stored at low temperatures, although better results may have been obtained than with nonlyophilized pollen stored at room temperature. KING (1959) found that there was a wide range of tolerance of loblolly pine pollen to freeze-drying. In his preliminary tests, freeze-dried pollen sealed in nitrogen gas maintained viability for as long as 99 days in an otherwise uncontrolled environment. Controlled pollinations, however, were not made with the treated pollen to determine its fertility, and this step is considered essential to the true measurement of pollen vitality. That a difference exists in the vitality required for germination of pollen and for actual formation of seed has long been recognized.

Other workers, HESSELTINE and SNYDER (1958), LIVINGSTON, CHING, and CHING (1962), JENSEN (1964), and most recently KING (1965), have reported attempted or successful preservation of pollen of a variety of species by freeze-drying or simple vacuum-drying.

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Procedure and Methods

In March, 1961, three lots of pollen were collected from individual Douglas-fir trees in isolated stands in MacDonald Forest near Corvallis, Oregon. The pollen was collected either by allowing sporangia on cut branches to shed pollen in the laboratory or by using a specially designed vacuum collector in the field. Each of the three lots was handled differently immediately after collection, and each was subjected to different treatments before freeze-drying:

1. Pollen of Lot 1 was freeze-dried immediately after collecting; moisture content of the greatly hydrated pollen was 16.2 percent at the time of treatment.
2. Pollen of Lot 2 was air-dried for 16 hours after collection to final moisture content of 6.3 percent, and then it was freeze-dried.
3. Pollen from Lot 3 was air-dried for 4 hours at room temperature to 8.5 percent moisture content after being collected in the laboratory, then it was prechilled at 0^0 C for 36 days before freeze-drying.

Hence the three lots of pollen, regardless of the method of collection, represented three pretreatments: Lot 1 was not air-dried or prechilled, Lot 2 was air-dried only, and Lot 3 was both air-dried and prechilled.

The apparatus for freeze-drying was a mobile Vir Tis Freeze-Dryer, Model 10-140. Quantities of pollen weighing about 1 gram were placed in lyophil tubes; all tubes to be freeze-dried were prefrozen for 20 minutes in the center well, which contained a coolant of acetone and crushed dry ice (solid carbon dioxide). This mixture created a temperature of about -78^0 C. After prefreezing the pollen, the tubes were withdrawn from the well and attached to vacuum ports for evacuation of moisture. The prefrozen pollen remained below 0^0 C for at least the first 5 minutes of vacuum-drying, during which time about 30 percent of the initial moisture usually was removed (CHING and CHING, 1964). Samples of each lot were evacuated in this way for periods of ½, 1, and 2 hours. The vacuum-drying phase was terminated by torch-sealing the necks of the lyophil tubes to keep the pollen under vacuum.

For future comparison with freeze-dried pollen, additional 1-gram quantities of pollen from each lot were placed in the tubes and sealed at normal atmospheric pressure after the pretreatments. Sufficient quantities of each lot of pollen were processed for storage at 20^0 C, 3^0 C, and -18^0 C and for subsequent over the 3-year period.

Germinability and moisture content were determined for each lot of pollen before freeze-drying and immediately after the treatment. Pollen germinability was estimated immediately after collection, after 1 year of storage, and after 2 years of storage by incubating the pollen at 30^0 C for 8 hours and then 20^0 C for 16 hours on the surface of an agar-cane sugar medium in a moist chamber. Freeze-dried pollen was not rehydrated, or subjected to any other recovery process, before testing germination. Viability or germinability was measured by the percentage of grains that had germinated at the end of 24 hours. Two slides for each treatment were prepared, and four fields, each encompassing from 50 to 100 grains, were sampled on each slide. Thus, between 400 and 800 grains were observed for each

test of germination for each treatment, with the difference between replications usually less than 5 percent. A grain was considered germinated if the tube cell had elongated from two to three times the original diameter (about 100 microns) of the ungerminated grain.

Ability of the freeze-dried pollen to set seed was determined immediately after treatment, then after storage, by applying the various lots to receptive ovulate cones under carefully controlled conditions. Pollinating bags isolated the female cones long before the period of receptivity and were not removed until after the period of pollen flight. In addition to applying freeze-dried pollen, fresh pollen from another tree was collected each year to serve as a control for comparison with the treated pollen. Wind-pollinated controls were compared with the experimental pollinations. In the fall of each year, cones were harvested and seeds extracted. Filled seeds in each treatment were counted, and the seeds were germinated to verify soundness.

The analysis of seed set was based on the percentage of sound seeds produced; only seeds that germinated and produced viable seedlings were considered to be sound. Production of sound seeds judged by this criterion gives essentially the same information as analyses based on the average number of sound seeds per cone.

Effects of Freeze-drying and Storage on Pollen Germination

Effects of freeze-drying, and temperature and duration of storage, on germination and moisture content are illustrated in *Figure 1* for pollen Lot 3. Pollen in Lot 3 was air-dried to 8 percent moisture content and prechilled after laboratory collection before it was freeze-dried. The results over the 3-year period are presented only for Lot 3 because its pretreatment was most favorable for retaining high germinability and fertilizing capacity for pollen after freeze-drying. Lots 1 and 2 showed similar results, except for differences that will be discussed.

No Storage:

Germination of pollen in Lot 3 before freeze-drying was 89 percent and moisture content was about 8.5 percent. Freeze-drying reduced viability and moisture content in proportion to time of vacuum-drying. Residual moisture content following the 2-hour treatment was only 2 percent, which probably represents chemically bound water. Germination of the pollen after 2 hours of vacuum-drying was still remarkably high at 72 percent, notwithstanding the extreme reduction in moisture.

Table 1. — The effect of pretreatment and freeze-drying on percentage of germination.

Vacuum duration Hours	Lot 1 Non-air-dried	Lot 2 Air-dried	Lot 3 Air-dried and prechilled
0*)	98	76	89
1/2	90	56	85
1	42	38	76
2	20	27	72

*) Not freeze-dried.

Comparison of all three lots after the 2-hour treatment makes clear that the lots differed in retaining viability in pollen as measured by percentage of germination *(table 1)*. Germination of Lot 3 was 81 percent of that for the unfrozen pollen, as compared to 20 percent and 36 percent for pollen from Lots 1 and 2. Lot 1, which was not air-dried, was reduced drastically in viability. Lot 2, which was air-dried but not prechilled, also showed considerable loss in viability after freeze-drying. Lot 3, however, which was both air-dried and prechilled, retained much higher levels of viability after freeze-drying. Thus, pretreatment of the pollen apparently constitutes an important step if the pollen is to be preserved by this technique. Prechilling in combination with air-drying apparently conditions the pollen so that, even after forced evacuation of water under low presssure to the bound-water level, the pollen grains still retain high viability. Such results have been confirmed on several lots of pollen treated the same way.

Storage:

After storage for 1 year at room temperature, only the freeze-dried pollen remained viable, while the untreated pollen had deteriorated completely *(figure 1)*. This finding was true in all three lots. In all tests, the residual moisture content associated with maximum viability lay between 2 percent and 5 percent. Thus, if viability of pollen is to be prolonged at room temperature, freeze-drying or some means of further reducing the moisture content is required.

The non-freeze-dried pollen from all three lots showed substantially higher moisture content than the freeze-dried

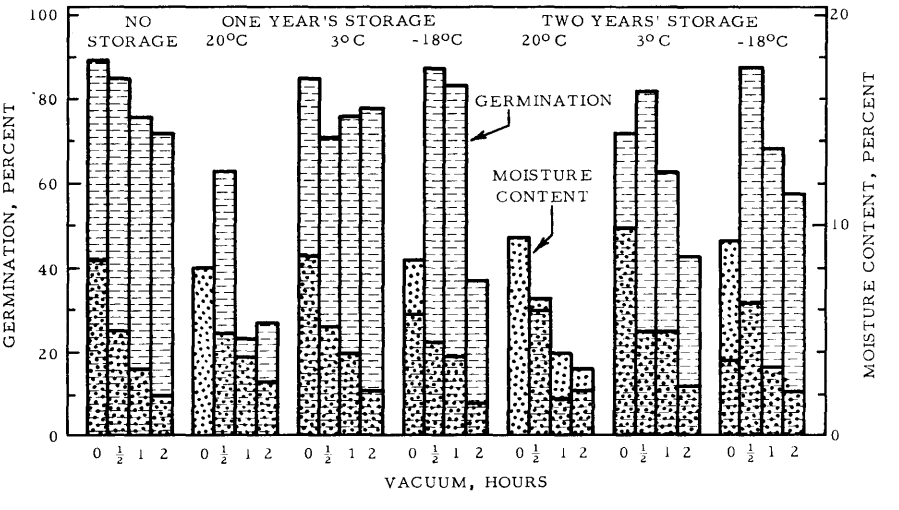

Figure 1. — Douglas-fir pollen, Lot 3 (air-dried and prechilled): Effect of freeze-drying, storage temperature and duration on pollen germination and moisture content.

samples, but still retained viability well following 1 year's storage at 3° C. The other levels of freeze-drying also resulted in high percentages of germination.

Pollen stored in the freezer was similar to pollen stored at room temperature in that under both conditions the freeze-dried pollen showed the highest percentages of germination. For example, at −18° C, the non-freeze-dried pollen showed only 28 percent germination, while the pollen freeze-dried for ½ and 1 hour showed values of 87 percent and 83 percent. These results indicate that 3° C was more favorable for high moisture levels in pollen, at which viability can be maintained, than was either 20° C or −18° C.

The same general results were noted following storage for 2 years, with the freeze-dried pollen showing the highest percentages of germination after storage at room temperature and in freezer, especially with the ½-hour level of treatment. Here again, 3° C was most favorable for maintaining the viability of non-freeze-dried pollen. Untreated pollen stored at room temperature was completely inviable, freezer-stored pollen was only 17 percent viable, and untreated pollen stored at 3° C showed 72 percent germination *(figure 1)*.

Effects of freeze-drying and storage on seed production

There were no apparent differences in percentages of sound seeds among the non-freeze-dried, freeze-dried, and wind-pollinated controls in 1961 when controlled pollinations were carried out soon after treatment *(figure 2)*. Freeze-drying did not materially impair the fertility of the pollen, especially at the short durations of freeze-drying, when time between treatment and pollination was short. Production of sound seed by both nontreated and treated pollen was about the same as production of seed by the two controls.

Following a year of storage, the percentage of sound seeds achieved through controlled pollinations with the treated pollen indicated that pretreatment, treatment, duration of storage, and temperature of storage all may affect vitality of pollen. Untreated pollen stored at room temperature produced practically no sound seeds. Only the freeze-dried pollen showed substantial production of seed after storage at room temperature for 1 year *(figure 2)*. The fertility of freeze-dried pollen was greater than that of untreated controls at all three temperatures of storage. In addition, pollen from two levels of freeze-drying and all three temperatures showed percentages of sound seed equivalent to, or greater than, those of the fresh pollen control, which itself was higher than 60 percent. Production of seed by the wind-pollinated control was low in 1962 because of loss from insects.

Following storage of the treated pollen for 2 years, there was a marked decline in fertility *(figure 2)*. No seeds were produced with untreated pollen regardless of storage temperature. No sound seeds were produced with pollen from any level of freeze-drying followed by storage at room temperature. Only freeze-dried pollen stored at 3° C and −18° C was still able to produce viable seeds following 2 years of storage. Production of seed by some samples was about as much as that of fresh pollen applied on the same tree, but was much less than that following only 1 year of storage. No explanation can be given for the low production of seed with fresh pollen in 1963.

Discussion

Fresh Douglas-fir pollen contained 12−16 percent moisture, which decreased to between 5 and 8 percent after 24 hours of air-drying, apparently because of loss of free water. Freeze-drying further reduced the moisture content proportional to duration of the drying period; following the 2-hour treatment, the residual moisture content was 2 percent or less. Accompanying the loss in moisture, there was a proportional reduction in viability of pollen. The magnitude of reduction was related not only to the duration of treatment but also to pretreatments of air-drying and prechilling before freeeze-drying. The process of denaturation during dehydration was the most likely cause of mortality. With non-air-dried pollen in a greatly hydrated condition, abrupt and forceful evacuation of water molecules under vacuum would tend to derange the spatial orientation of the internal structure of various macromolecular species in an irreversible fashion, resulting in death. The process of freeze-drying was not a simultaneous action of freezing and drying, and presumably the sudden removal of water to the chemically bound level was the action to which loss of viability can be attributed.

That drying and not freezing was the cause of reduced viability is attested to by information derived from supplemental tests. Freezing and drying were conducted simultaneously, and viability and moisture content were unaltered after treatment. After simultaneous freezing and drying, germination remained the same and moisture level in the pollen was still high. Thus, freezing in itself is not believed

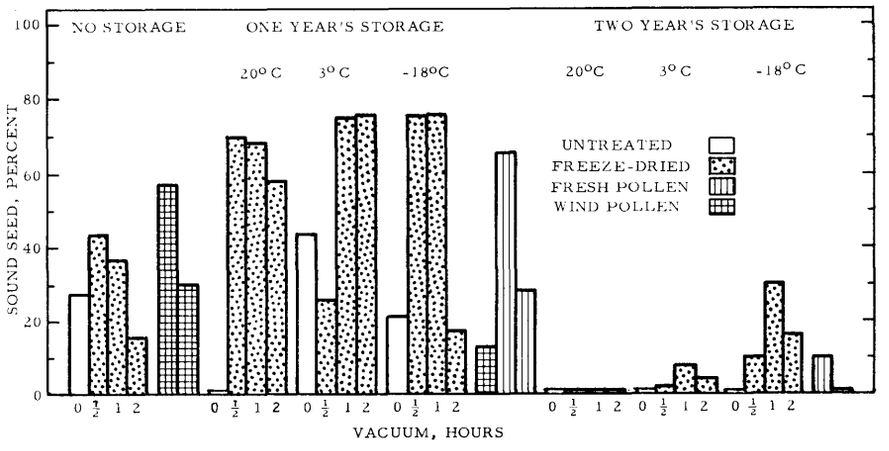

Figure 2. — Percentages of sound seed formed with freeze-dried and non-freeze-dried Douglas-fir pollen of Lot 3, which was stored 0, 1 and 2 years at three temperatures.

to damage the pollen when the exposure time at −78° C does not exceed 20 minutes. In further tests air-dried and non-air-dried pollen were immersed in liquid nitrogen at −196° C. Viability was unchanged unless the pollen contained over 20 percent moisture, when it was killed outright. Air-dried Douglas-fir pollen is generally resistant to short exposures to subfreezing temperatures.

From the data, there appears to be a positive one-way correlation between germination in vitro and production of seed in vivo. In no instance did pollen that was totally inviable as measured by germination in vitro set a substantial number of sound seeds. For example, untreated pollen that failed to germinate after storage at room temperature produced no seed. However, higher viabilities of pollen do not necessarily mean high set of seed. There were many samples of pollen where values for germination as high as 80 percent resulted in low production of seed. For such results the most plausible explanation is that different degrees of vitality bring about germination in vitro and formation of seed in vivo. There apparently is a threshold beyond which vital processes may be sufficient to bring about germination in the laboratory but are insufficient to permit extended growth of a pollen tube, completion of microgametogenesis with discharge of the sperm cells, and subsequent fertilization.

Increase in the United States of large-scale application of pollen to receptive flowers on trees in seed production areas and seed orchards by mist blowers indicates need of improved knowledge and technology for preserving and safely storing tree pollen in large quantity. Equipment companies have already placed on the market freeze-drying apparatus of different designs that is able to process large batches of pollen in virtually any type of containers, such as serum bottles, Petri dishes, or trays. With serum bottles, for instance, pollen is placed unfrozen in the bottles. Special split rubber stoppers are partially inserted in each bottle, and the tray containing the bottles is placed on the mechanically refrigerated process shelf. Pollen then will be efficiently frozen, freeze-dried, and stored in the bottles under the original vacuum by means of a stoppering plate that firmly seats the rubber stoppers in the bottles.

In conclusion, research in pollen storage so far has indicated that extending pollen longevity and fertility requires not only improved techniques but also further understanding of consequences in freeze-drying process so as to reduce costly loss in this important step in tree improvement.

Summary

Three lots of pollen collected in the spring of 1961 were freeze-dried following pretreatments of no air-drying, air-drying, and air-drying in combination with prechilling. The pollen freeze-dried for periods of 0, ½, 1, and 2 hours, after which the pollen was stored under vacuum in lyophil tubes at temperatures of 20° C, 3° C, and −18° C. Viability of pollen and moisture content were determined at each step. Tests of pollen germination in vitro were made on a nutrient medium immediately following treatment, after subsequent storage for 1 year, and after 2 years' storage. Samples of pollen from all treatments were applied to receptive ovulate cones under controlled conditions in 1961, 1962, and 1963 to determine their ability to produce seeds.

Germinability of the pollen was reduced by freeze-drying, and the magnitude of reduction was related to pretreatment of the pollen as well as to duration of the vacuum. Air-drying followed by prechilling at freezing temperatures significantly lessened the reduction in viability caused by freeze-drying. Freeze-drying also reduced the moisture content to as low as 2 percent with the 2-hour treatment.

In general, after storage for 1 and 2 years at 20° C, 3° C and −18° C, freeze-dried pollen retained fertility to a higher degree than did pollen not freeze-dried. Retention of fertility because of freeze-drying was enhanced when the pollen was air-dried and prechilled before freeze-drying. With such pretreatment, fertility of pollen as measured by percentages of sound seed after 1 year's storage at three different temperatures was equivalent to or greater than that of fresh pollen. Freeze-drying reduced moisture content of the pollen to low levels at which preservation can best be assured. One-half or 1-hour vacuum periods were sufficient to insure viability, but 2 hours' drying resulted in excessively reduced viability.

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