EXTRACTION AND STORAGE OF LOBLOLLY PINE (Pinus taeda) POLLEN

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Abstract.--Five years of investigations at North Carolina State University on extraction and storage of loblolly pine pollen have revealed the following: (1) the most critical factor in pollen storage is keeping the catkins and pollen dry during the extraction process; (2) pollen dried to a moisture content of 8 to 10 percent will store satisfactorily for two to three years with most of the methods investigated; (3) the vacuum method of pollen storage is effective for up to three years and yields higher and more consistent viability than do the conventional methods tested. Justification of this expensive method is still in question at this time.

Additional keywords: Moisture content, vacuum storage, germinability, catkins, clonal variation.

Due to the fact that it takes one to two weeks to collect and extract southern pine pollen, the use of pollen for control-pollinations during the same year of collection is very difficult. Therefore, pollen storage for use of pollen in subsequent years has been employed by tree breeders. To insure the highest viable pollen for control-pollinations, proper extraction and storage procedures must be determined. In the past the North Carolina State University-Industry Cooperative Tree Improvement Program has advised its members to extract pollen and dry it over a desiccant (usually LiCl) under refrigerated conditions at approximately 35°F (2°C). However, success with this method seemed rather sporadic. At the same time, the Cooperative decided to establish a breeding pollen bank at North Carolina State to facilitate the control crossing in the advanced-generation breeding. Therefore, it was decided to initiate investigations to determine the best methods of pollen extraction and storage for insuring the most viable pollen. Investigation began in the spring of 1973. This paper reports observations and results from investigations in four areas: (1) pollen extraction; (2) pollen storage; (3) effectiveness of vacuum for protection of pollen during transit; and (4) effectiveness of vacuum for protection of pollen at room temperature. After two years the vacuum method was better for storage than the checks but the difference wasn't of practical significance. Experiments 3 and 4 were, therefore, set up to determine if there was a real advantage in the vacuum method.

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Throughout the studies, pollen quality was evaluated by the standard North Carolina State germinability test. The pollen is germinated in a .01 percent sucrose solution for 48 hours at approximately 70°F (21°C), Percent germination is based on the number of germinated pollen grains in a 200 count. A grain is counted as germinated when the tube length is equal to or greater than the width of the pollen grain. Table 1 describes the system used to rate the pollen as to its usefulness for effecting control-pollination.

Table 1.--Rating used to determine usefulness of pollen for effecting control-pollinations

Percent Germination	Rating	Utility for Control-pollinations
35+	Excellent	Use
20 - 34	Good	Use
10 - 19	Fair	Use only if fresh pollen unavailable
1 - 9	Poor	Don't use
0	Very poor	Don't use

POLLEN EXTRACTION

The most efficient pollen extractor will be one designed to extract the pollen and dry it to a sufficiently low moisture content with the shortest exposure to room temperature. This is critical because pollen viability during storage is greatly affected by moisture content and temperature of the pollen. Pollen will quickly lose viability unless stored under near-freezing temperature. Excess moisture in pollen (especially in combination with heat) leads to molding. In fall, 1972 an extractor was constructed taking these factors into account, patterned after a smaller one constructed by the North Carolina Forest Service (Summerville and Turner, 1973). Twenty-four 12" (.top diameter) gasoline funnels were modified so that the pollen from catkins placed in the funnels would collect in glass bottles attached to the bottom of the funnels. Room temperature air was blown into each bottle and escaped through the funnel, facilitating and speeding up the drying process. The 24-unit extractor was placed in a temperature-humidity control chamber, set at 70 - 80°F (21 - 27°C) and a relative humidity of about 40 percent. Equilibrium moisture content of pollen under these conditions is about 8 - 10 percent, which is sufficiently low for prevention of mold during storage. Catkins of the requested clones have been collected and shipped to North Carolina State by Cooperative members for five years now.

Many observations made during the past five years concerning the correlation between extraction and viability are:

1. Condition of catkins upon arrival has a significant effect on extraction and viability. Unripened catkins shed very little pollen, regardless of the duration of extraction. Yield of pollen from ripe, wet catkins is poor and the pollen is prone to mold. Best yields and viability of pollen are obtained from ripe, dry catkins. Best conditions for transporting

catkins to assure good yield and viability are created by collecting them when they are ripe and predrying them two to three days before shipping.

2. If mold starts in the catkins or pollen during shipment or extraction, the chances of the pollen storing well are greatly reduced.

3. The most important element in successful pollen storage is proper extraction whereby the catkins and pollen are kept dry at all times and can be moved quickly into cold storage.

Based on these observations, the following recommendations are made for collection and shipment procedures:

1. Ideally the best time to collect catkins is at the first sign of pollen shedding. However, this is a very dangerous stage as all the pollen could fly within a couple of days or within several hours if the weather conditions are right. Therefore it is desirable to collect the pollen at an earlier stage, perhaps when the bases of the catkins just start to break open or when they become yellow but produce very little liquid when squeezed between the fingers.

2. Preparation of catkins for shipping is one of the most critical aspects of pollen handling. If improperly packed, the green, moist condition of the catkins causes heat buildup and mold very quickly. Once molding has begun, the pollen is not any good. Several steps should be taken to avoid this problem.

   a. Collect catkins by clusters instead of individual catkins. This allows more aeration and less packing.

   b. If catkins are still very green, put them in paper bags (not over one-fourth to one-half full), hang them in a dry room and circulate air around them by means of a fan for a couple of days before shipping. This process will drive some of the moisture off the catkins, thereby reducing the chance of molding during shipping.

   c. When preparing for shipping, line paper bags with paper towels for absorption of excess moisture. Make only two to three narrow folds at the top of the bag and securely staple it closed. Never roll or fold the top of the bag down to the point that the catkins are tightly packed together and have no "breathing room."

   d. The paper bags should be loosely packed in a cardboard box with newspapers in between them. Holes three inches square (76 mm square) should be cut in the box for aeration.

   e. Sausage casing is less desirable for shipping catkins than paper bags. These casings are more conducive to heat and moisture buildup than are the paper bags.

POLLEN STORAGE

In establishing a research pollen bank it is necessary to use a method that will allow for successful storage of pollen for five years or longer. This option decreases the need to collect pollen each time it is needed, and it serves as insurance for gene conservation if disaster should strike the parent. In evaluating the options, it was determined that freezing and vacuum storage had been used for long-term storage of pollen (Hermann, 1969; Ching and Ching, 1964; King, 1961), and that pollen had been flame-sealed under vacuum (Jensen, 1970). Using these leads, a flame-seal vacuum device was constructed at North Carolina State based on a similar device being used at the time to store Cronartium fusiforme aeciospores. The system was put into use in spring, 1973 for storing the pollen in the pollen bank and at the same time a study was initiated to test the effectiveness of the vacuum system. The following conventional methods of pollen storage were used as checks for the vacuum storage study: (1) stored in air-tight bottles in refrigerator at 35°F (2°C); (2) cotton-stoppered bottles in refrigerator at 35°F (2°C); and (3) cotton-stoppered bottles inside desiccator (over LiCl) refrigerated at 35°F (2°C). Pollen from three clones with viability in excess of 66 percent and moisture content of 6 - 9 percent were chosen for the study. Two new methods of storage were tested. They were (1) vacuum storage under refrigeration at 35°F (2°C), and (2) vacuum storage under freezing conditions at -9°F (-20°C).

Equal amounts of pollen from each clone were placed in thirty-two 2-ml ampules (32 ampules/clone x 3 clones = 96 ampules) on April 6, 1973. The ampules were flame-sealed under a vacuum of approximately 38-mm Hg. and then placed in storage. Germination tests were made at 3, 6, 12, 18, 24, 36 and 48 months. Table 2 lists the average germination by treatment after three and four years of storage.

Table 2.--Germination (percent) of pollen stored by five different methods after three or four years of storage

Method of Storage	Percent Germination	
	Third Year	Fourth Year
Vacuum storage at 35°F (2°C)	64	29
Vacuum storage at -9°F (-20°C)	69	29
Rubber-stoppered bottle at 35°F (2°C)	15	15
Cotton-stoppered bottle at 35°F (2°C)	22	18
Cotton-stoppered bottle in desiccator at 35°F (2°C)	4	0

At the end of the third year, pollen under the vacuum had shown no noticeable decrease in germination and had stored much better than any of the checks. There was very little difference between the freezer and refrigerator treatments. Germination results from the checks were rather inconsistent and much poorer. Part of the poorer results of the checks may possibly be due to an inadvertent bias in that each check bottle was exposed to a sudden temperature change at each assessment while a new vial was available each time for the vacuum ampules.

Fourth-year results reflected an important decline in germination for every storage method. Essentially all of the check pollen was dead, with the exception of clone B which tested 46 percent germination with both the rubber-stoppered check and the cotton-stoppered check (Table 3). There was also an important decline in germination with the vacuum method except for the same clone which maintained 56 (62 and 50) percent average germination; the other clones averaged 18 (17 and 18) and 14 (10 and 18) percent. These results indicate a strong clonal variation in storage ability of pollen. Clone B stored the best in all treatments except for the desiccator treatment in which all pollens were dead after four years. This method proved the least effective of all. Average germination of the two vacuum stored treatments (freezer versus refrigerator) was the same after four years (29 percent). The 29 percent germination is very sufficient for making controlled crosses; however, the 29 percent is not actually representative but is a reflection of the unusually high value of clone B (56 percent) versus the low values of 18 percent and 14 percent for the her two clones. Germinations of the latter two clones are still acceptable for making control crosses but would be rated "fair" and "use only if fresh pollen is not available."

Table 3.--Clonal variation in germination after four years of storage by five different techniques

	Percent Germination			
Method of Storage	Clone A	Clone B	Clone C	$\bar{X}$
Vacuum storage at 2°C	17	62	10	29
Vacuum storage at -20°C	18	50	18	29
Rubber-stoppered bottle at 2°C	0	46	0	15
Cotton-stoppered bottle at 2°C	9	46	0	18
Cotton-stoppered bottle in desiccator at 2°C	0	0	0	0
Clonal Average	9	41	6	

The sudden decline between the third- and fourth-year assessments of the vacuum method was unexpected. The decline indicates one of two things: (1) vacuum storage of loblolly pine pollen under the conditions we used is only effective for three years of storage, and (2) there was a sampling error or an error in the 1977 viability tests. The study will be continued one more year and the final assessment should tell if there is validity to alternative #2. It will be remembered that separate ampules are sampled in different years, so aberrant results within one year will have no effect on those of another year.

EFFECTIVENESS OF VACUUM FOR PROTECTION OF POLLEN DURING TRANSIT

In summer, 1975 a study was conducted to determine if vacuum storage would afford good protection of pollen during transit, as suggested in the literature (Wilcox, 1966) One vacuum ampule and one screw-cap bottle (nonvacuum) containing pollen of each of three clones were sent by first-class mail from Raleigh to three cooperators, one located in Georgia, Alabama and Tennessee. The pollen

used was excess from the spring, 1975 collection and had been stored under refrigeration in air-tight bottles until time of the study, when part of them were sealed under vacuum. The pollens were packaged in polyfoam mailers (normally used for shipping blood samples) on June 30, 1975. Upon receiving the pollen, each cooperator returned them to Raleigh. The pollens arrived back in Raleigh after being in transit for a total of 9 - 10 days. Germination tests run before shipping and upon return to Raleigh are reported in Table 4.

Table 4.--Initial germination (percent) and final germination of pollen of three clones after nine to ten days in transit

Clone	Initial Percent Germ.	Final germination (%) upon arriving back in Raleigh					
		Georgia		Alabama		Tennessee	
		Vial	Ampule	Vial	Ampule	Vial	Ampule
20-506-1	47	30	52	55	61	29	34
11-61	36	0	0	0	0	0	0
8-526-4	52	34	26	63	65	36	49

Surprisingly there was little difference in germination between pollen shipped in bottles and pollen in the vacuum ampules, as both afforded good protection to the pollen. Two of the clones stored very well under both treatments, while 11-61 (initial germination of 36 percent) returned dead in all cases. Probably the 11-61 pollen was in a weakened condition at the beginning of the study, due to some detrimental environmental conditions during collection, shipping, or extraction. Possibly mold got into the pollen during prestorage treatments but was retarded by the low storage temperature, only to be reactivated again as soon as it was removed from refrigeration. in a couple of instances for the other two clones, the final germination was higher than the initial. However, this does not represent an increase in viability of the pollen bur is due to experimental error in the sampling technique.

A possible explanation for the unexpected similarity between the two methods would be the effectiveness of the polyfoam containers in protecting the pollen from heat buildup. To determine if the polyfoam containers were masking differences between the two methods, another test was conducted wherein the pollen was subjected co room temperatures without use of the polyfoam containers.

EFFECTIVENESS OF VACUUM FOR PROTECTION OF POLLEN AT ROOM TEMPERATURE

This test was designed to determine the effects of continuous room temperature storage on the germinability of pollen stored in screw-cap bottles and in the vacuum ampules.

Pollen from three clones was chosen for this experiment, based on germination tests conducted a few days prior to initiation of the study. The pollen used was excess from the spring, 1975 collection and had been stored in air-tight bottles under refrigeration until the time of the study. Average pollen germination for the three clones at the start of the study was 63 percent.

The experiment ran for eight weeks. Eight vacuum-sealed ampules and eight air-tight (nonvacuum) bottles of pollen from each clone were prepared on July 22, 1975 and were placed in a room where the temperature averaged 80°F (27°C). One ampule and one bottle of pollen from each clone were tested for germination each week during the eight-week period. Germination results are recorded in Table 5.

Table 5.--Average[1]/ weekly germination of pollen stored in air-tight bottles and vacuum-sealed ampules under room temperature of 80°F (27°C) for eight weeks

Assessment Time	Air-tight Bottle	Vacuum Ampule
First week (July 29)	54	60
Second week (August)	21	40
Third week (August 12)	35	53
Fourth week (August 19)	8	27
Fifth week (August 26)	0	45
Sixth week (September 2)	1	10
Seventh week (September 9)	0	12
Eighth week (September 16)	0	18

1/ Average of the three clones

The vacuum-sealed pollen rated higher in germination each week. After the fifth week, pollen stored in the bottles was essentially dead. The vacuum-sealed pollen dropped noticeably in germination between the fifth and sixth week but still rated fair through the end of the eighth week, at which time it tested at 18 percent germinability, which is still high enough to consummate control-pollinations. Germination percent for the pollen under vacuum actually showed an increase from the sixth week to the eighth (10, 12, 18); however, as mentioned earlier, this type variation is due to experimental error in the sampling technique. These results indicate that under extreme temperature conditions the vacuum ampules definitely afford the best protection.

SUMMARY AND DISCUSSION

During the past five years, experience in pollen extraction and storage has taught us that the most critical factor in maintaining viable pollen is to control its moisture content. Pollen stored at a 10 percent moisture content maintained sufficient viability for up to three years for most of the methods tested. Pollen stored at a moisture content greater than 10 percent has the propensity to mold. Mold often occurs in pollen during the process of moisture content reduction brought about by a desiccant such as lithium chloride. To avoid the problems, the recommended procedure is to reduce the moisture content of the pollen during the extraction process. It is preferable to keep the pollen in the extractory a few days longer than is normal, to assure the proper moisture content level of about 10 percent.

Results from these studies indicate that the vacuum method of pollen storage has definite advantages over the nonvacuum methods. The pollen under vacuum stored more consistently for three years than the check pollen. Part of the difference in germination between the vacuum-stored pollen and the checks could be due to an experimental bias against the checks. Further studies will be carried out to determine if in actuality this bias exists. If the pollen can be stored in air-tight bottles for three years with good germination, the vacuum method may not be needed. The ampules offer no advantage in shipping as the polyfoam packers afford good protection to the pollen, regardless of how it is stored. In the meantime the North Carolina State Cooperative will continue to use the vacuum method to assure the best pollen quality possible, as this method has produced the most consistent results.

It was hoped that vacuum storage would be effective for long-term storage of pollen (3+ years), but the fourth-year results tend to dispel that hypothesis. The fifth-year (final) assessment should tell if an error was committed in the 1977 assessment. If germination is low next year, then the vacuum storage effectiveness will be about three years. If germinations are higher next year, then perhaps there was some error in the test method at the fourth year; and perhaps the vacuum method will be effective for more than three years. Whatever the final results indicate, it would be difficult to justify the expensive vacuum method for normal pollen storage by an individual industry. It would be more readily justified for a research pollen bank like the one at North Carolina State University.

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