

## BLACK WALNUT POLLEN STORAGE AND GERMINATION

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Abstract.--Controlled pollination in forest trees often necessitates pollen storage for long periods. Early studies at Purdue University on black walnut pollen storage techniques demonstrated that refrigerator storage without dessication provides successful short-term storage (1 to 3 weeks), and later studies showed that storage in liquid nitrogen for several years was possible. Pollen germination of 24 clones revealed that germination of fresh pollen was 31.5% compared with 29.8% for pollen stored one year in liquid nitrogen. Germination fell to 23.1% and 13.3% respectively following two and three-year-storage. Pollen from the few clones available for germination tests after four, five, and six years storage in liquid nitrogen tested 58, 51, and 54% respectively. Pollen stored in liquid nitrogen for one year affected successful controlled pollinations with seed set and seed germination percentages as high as that obtained from fresh pollen.

### INTRODUCTION

As programs for the genetic improvement of black walnut (*Juglans nigra* L.) advanced, it became necessary to investigate facets of control pollination in order to acquire the capability to maximize genetic gain. The paucity of information available on storage and viability of black walnut pollen led to this study.

Pollen storage is necessary primarily due to the wide variability associated with flower phenology. Unless pollen is successfully stored, certain specific crosses can not be made. For example, when pollen of one source matures a week in advance of pistillate receptivity, some means of short-term storage is needed in order to make that specific cross. If on the other hand, the situation is reversed, and the pistillate flower is receptive a week in advance of pollen maturation, the pollen would have to be stored for one year. Both of these situations occur in black walnut. In addition, maximizing genetic gain from seed orchards through mass supplemental pollination will require successful storage.

Storage of walnut pollen has been considered difficult, at least until recently. Griggs et al. (1971) stored Persian walnut (*Julans regia* L.) pollen for a year at  $-19^{\circ}\text{C}$  with no adverse effects. Hall and Farmer (1971) proved

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To maintain pollen viability after removal from liquid nitrogen, the vials of pollen were immediately submerged in a 30° C water bath. This sudden **rise in** temperature is necessary to prevent moisture from crystalizing inside the pollen grain, which occurs between 0 and -20°C (Meyer and Anderson, 1952). At this point, the pollen was ready for control pollinations. If not used that day, it was stored in a standard refrigerator at approximately 4 C.

#### Pollen viability

Techniques and media described by Hall and Farmer (1971) were used to test pollen viability. The most desirable media for our purposes was found to be 200 gms. sucrose, 0.3 gms. boric acid and 6 gms. bacto agar in 1000 ml of water, heated until all agar flakes were dissolved, and the mixture cleared. Approximately 10 ml of the media were distributed to standard-size petri dishes, allowed to cool, then stored in a refrigerator.

In 1974, significant staminate flower production occurred in our clone bank at Martell Forest near West Lafayette, Indiana, and a study of pollen germination of 51 clones was undertaken. A random sample of mature catkins was collected in the afternoon, and allowed to dehisce overnight (18 to 20 hours) in a growth chamber maintained at 27°C and 40 percent relative humidity. The following morning a random sample of pollen was dusted on previously prepared medium. Twenty-three hours later, pollen germination was recorded. The germination percent was based on random sample of 250 pollen grains.

Considering the possibility that there could be variation associated with the time germination took place, a small study was set up to determine the period of maximum germination. Six clones were utilized in the study, and germination counts were made at 4, 10, and 23-hour intervals. Length of time fresh pollen remained viable at room temperature (23 C) was tested using these same clones by checking germination at 12 hour intervals until pollen no longer germinated on fresh medium.

#### Control pollination with stored pollen

The ultimate test of pollen viability after long-term storage in liquid nitrogen was the utilization of stored pollen in making control pollinations. Pollen stored for one year was utilized in making controlled crosses in 1972 and 1975. Pollination techniques and equipment were described by Beineke and Masters (1976).

### RESULTS

#### Pollen storage and viability

Refrigerator storage, without desiccation, provided satisfactory short-term storage for one to three weeks (Table 1). Freezer storage and desiccator treatments were inconsistent and for the most part damaging. Black walnut pollen too moist for storage without desiccation is usually dead upon extraction. Immediately after extraction walnut pollen occasionally appears to be moist; i.e., it is dark yellow, does not flow freely, and tends to clump or cling together. Pollen in this condition is usually dead or has very low germination, and desiccation does not revive it. Moisture content of fresh pollen varies from 10 to 30 percent. Generally, moisture content above 15 to 20 percent shows the visual symptoms of moist pollen and will not germinate.

Table 1.-- Germination of 3 sources of black walnut pollen stored under 4 different refrigerator and freezer conditions.<sup>1/</sup>

Storage treatment <sup>2/</sup>	Percent pollen germination		
	Source 82	Source 102	Source 118
Fresh	18.4	48.1	50.5
After one week			
Refrigerator- without desiccation (4 C)	16.5	19.6	36.2
D6R	4.6	4.1	6.8
D12R	8.7	6.4	19.0
D24R	7.9	24.2	26.0
Freezer-without desiccation (-15 C)	6.4	3.0	5.0
D6F	1.0	2.1	1.0
D12F	1.0	2.5	4.8
D24F	1.0	1.0	2.4
Room temperature (21-24 C)	0	0	0

<sup>1/</sup>Data from the two refrigerators were not significantly different and were averaged

<sup>2/</sup>The symbolism refers to the hours pollen in a refrigerated desiccator (D), then taken out and stored in a refrigerator (R), or freezer (F).

Liquid nitrogen storage was successful for most clones for one year, and in fact, pollen germinability of some clones appeared to increase after storage (Table 2). This is probably due, however, to a difference in the condition of the germination medium used in different years. In terms of overall means, fresh pollen germinated 31.5 percent vs. 28.9 percent for pollen stored one year. After one year's storage, germination fell to 23.1 and 13.3 percent at 2 and 3-years, respectively. Germination of the few pollen sources available for testing at 4, 5, and 6 years, while much higher than expected, represent a small sample of sources that have already demonstrated their viability after at least one year's storage (Table 2). In 1974, the thorough fresh pollen germination study of all 51 clones available for testing produced an average of 35.7 percent germination which compares favorably to the 31.5 percent for clones stored in several different years. Pollen germination varied from 1 to 73 percent in the 51 clones in the 1974 test.

Pollen germination within clones can vary radically from year to year. For example, fresh pollen from clone 31 germinated 10, 60, and 51 percent in 1971, 72, and 74, respectively (Table 2). However, some variation is probably due to media differences, counting techniques, timing of counting, and conditions of the catkins upon collection between years rather than true differences.

Table 2.-- Germination percent of fresh and liquid nitrogen stored black walnut pollen.

Clone no. and yr. collected	Fresh pollen <sup>1/</sup>	After liquid nitrogen storage					
		1 year	2 years	3 years	4 years	5 years	6 years
1-71	10	25					
4-71	0	30					
9-71	20		18				
21-71	15	25					
31-71	10	20					
31-72	60	20	0				
31-74	51	60	60				
34-71	50		1				
36-72	90		50			51	
36-74	45	25		25			
44-74	13	0					
48-71	15	30					
48-74	20	0					
49-71	10	10					
49-74	15	60	50	12			
55-71	40		1	10			
55-74	16	0					
71-71	10	5					
72-71	10		50				
82-71 <sup>2/</sup>	60	50		1	58		47
82-71	60	40		31			61
96-71	10	30					
102-71	50	50	0				
118-71	75	70	1	1			
Mean % Germ.	31.5	28.9	23.1	13.3	58.0	51.0	54.0

<sup>1/</sup>Germination after the 16 to 24 hour extraction period.

<sup>2/</sup>Pollen was taken from a syringe used for control-pollination in 1971 and has been thawed and replaced in storage 4 times.

As indicated earlier, some pollen sources stored in liquid nitrogen for more than one year were still viable. Even more remarkable, one vial taken from a syringe used for control pollinations in 1971, thawed, then replaced in liquid nitrogen stored 4 different years, germinated nearly as well in 1977 as it did fresh in 1971 (Table 2).

Liquid nitrogen storage of black walnut pollen was successful; however, germination of pollen placed in refrigerator storage after initial liquid nitrogen storage is considerably reduced after 24-hours in refrigeration. In a 7 clone test, pollen germination was 31 percent after one year in liquid nitrogen and the same pollen germinated only 13 percent after 24 hours in refrigerator storage.

Maximum pollen germination occurred in most clones four hours after being placed in the medium (Table 3). The drop in pollen germination percent over time, was due to pollen tube disintegration on the medium. Although clone 7 increased germination after four hours, additional experience since the data was collected, show that the time interval expressing maximum germination for most clones is in the six to eight hour range. If germination counts are delayed to the 23-hour period, disintegrated pollen tubes and fungus or bacterial growth often obscure live tubes and make counting difficult.

Table Germination percent of 6 fresh black walnut pollen sources at 3 time intervals.

Clone No.	4 hours	10 hours	Net change (4-10 hours)	23 hours	Net change (10-23 hours)
86	24	20	-4	20	0
101	24	22	-2	21	-1
7	30	50	+20	42	-8
18	20	8	-12	10	+2
108	25	16	-9	17	+1
55	23	17	-6	16	-1
Mean	24.3	22.2	-2.2	21.0	-1.2

The final aspect of pollen viability information needed was the duration of viability at room temperature. In a small study using the clones in Table 3, pollen remained viable up to 96 hours at 23 C. The fact that pollen of all clones tested remained viable for at least 24 hours indicated there would be no problems with pollen viability during controlled pollinations.

#### Control pollination with stored pollen

Pollen stored in liquid nitrogen for one year affected successful controlled pollination in 1972 and 1975 (Table 4). Seed originating from stored pollen matured and developed normally, and seed set utilizing stored pollen was as good as that obtained from fresh pollen. In fact stored pollen source 31 produced double the seed set as compared to fresh pollen from source 31. Overall, stored pollen produced a slightly higher percentage of mature seed, 29 vs. 25 percent, than fresh pollen. Germination of seed which resulted from stored pollen was nearly the same as from fresh pollen (Table 4).

Table 4.--Success of control pollination using nitrogen stored pollen.

Year	Pollen source	No. flowers pollinated	No. mature seed set	Percent seed set	Percent seed germination
1972	102-stored 1 year	210	44	21.0	45.7
1972	Fresh pollen	599	143	23.9	47.2
1975	31-Stored 1 year	50	32	64.0	41.4
1975	31-Fresh	59	18	30.5	38.9
1975	Fresh pollen all sources	514	136	26.5	41.0
1972 & 1975	Stored	260	76	29.2	43.8
1972 & 1975	Fresh	1113	279	25.1	44.3

#### CONCLUSION

This long term study has shown that it is possible to store black walnut pollen for long periods and use it successfully in control pollination to obtain seedlings of known parentage. Problems similar to those encountered with black walnut pollen storage will occur with other hardwood species and hopefully some of the solutions that apply to black walnut will also remedy problems with other hardwood species.

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