PINE POLLENS FROZEN FIVE YEARS PRODUCE SEED

by R. Z. Callaham and R. J. Steinhoff1

Deep-freezing of pine pollen offers a means of prolonging its storage life. Early work showed that pollen could be frozen without losing its viability. A study was started in 1958 at the Institute of Forest Genetics at Placerville to determine how long frozen pollen of several pines would remain viable. This paper reports *in vitro* germination and *in vivo* seed production by pollens stored at -20°C. up to 5 years.

References to low temperature storage of forest tree pollens are infrequent in the literature. Boden (1958) successfully stored eucalyptus pollen at −16°C. for 7 months. Duffield and Callaham (1959) reported success in freezing pine pollen for 1 year at -23°C. They found that fresh and stored pollen were about equally effective in producing sound seed. Wright (1959) mentions good results using pollen stored for 1 year at -18°C. Ehrenberg (1961) reported results of crossing experiments with pollen stored for 3 years in a freezer. Her *in vivo* studies with *Pinus sylvestris* showed that storage at +4°C. was much less effective than storage at -18°C. After 3 years, pollens stored at +4°C. and -18°C. both germinated nearly 100 percent *in vitro.* Crossings using the pollen stored at +4°C. did not produce any filled seed, but crossings using pollen stored at -18°C. gave only slight reductions in seed-set and produced about half as many filled seed as would be expected from fresh pollen.

Methods and Materials

The study was initiated in 1958 with the following species: *Pinus jeffreyi* Grey. & Balf., P. *monticola* Dougl., P. *contorta* var. *murrayana* (Grey. & Balf.) Engl., *P. ponderosa* Laws., and P. *sabiniana* Dougl. For each species three trees that flowered early were selected to serve as pollen parents and three that flowered late were selected as seed parents. All trees were located on a transect through the Sierra Nevada near Placerville, Calif.

In the spring of 1958, ripe pollen was collected and extracted, using the technique described by Cumming and Righter (1948). Pollen moisture content was not controlled, but pollen was dried to the point where it flowed.

Pollen from each tree was divided into several small aliquots in small screw-cap glass bottles. Each aliquot contained enough pollen to pollinate the conelets in 9 pollination bags. One aliquot was stored at room temperature until used from 2 to 10 days later. Two aliquots were placed in a domestic refrigerator kept at about 5°C. to be used after 1 and 2 years of storage. Nine aliquots were placed in a domestic deep freezer kept at −20°C. The first aliquot from the deep freezer was used from 2 to 10 days later when female parents were ready to be pollinated. The remaining aliquots were to be used after 1, 2, 3, 5, 7, 10, 15, and 20 years of storage. In each test year, fresh pollen for comparison was again collected from the original pollen parents. All pollens were tested for viability by the method of Righter (1939). Pollens removed from storage were kept at room temperature until pollinations were completed.

Fresh and stored pollens were used in controlled pollinations (Cumming and Righter 1948) on each seed parent in 1958, 1959, 1960, 1961, and 1963. On a seed parent tree each pollen aliquot was used to pollinate the strobili isolated in three pollination bags. Ripe cones were collected, and seeds were extracted. Filled and hollow seeds were counted after separation by winnowing. A sample of hollow seeds was examined for insect damage. All infested seeds were presumed to have been filled, and numbers of filled and hollow seeds were corrected accordingly.

Results and Discussion

All desired crosses to determine ability of stored pollen to produce seed *in vivo* could not be completed. Some species had sporadic cone production or very low seed set from controlled pollination. Squirrel depredations eliminated crosses on some species. One tree adorned with pollination bags was felled. Only the results for *P. monticola* were complete. The next best results were for P. *jeffreyi.* Only data for these two species warranted statistical analysis. Fragmentary data for the other three species are discussed wherever they provide useful information.

Brief freezing of pollen for 2 to 10 days immediately following extraction increased yield of filled seeds (figs. 1 and 2). For P. *jeffreyi* the increase was large and statistically significant; for *P. monticola* it was slight. In contrast, fragmentary data for P. *sabiniana* showed that fresh pollen gave

1At the time this research was done, both authors were at the Institute of Forest Genetics, Pacific Southwest Forest and Range Experiment Station, Forest Service, U. S. Department of Agriculture, Placerville, Calif. Callaham now is Chief, Branch of Genetics, Division of Timber Management Research, U. S. Forest Service, Washington, D. C. Steinhoff now is at the Forest Science Laboratory, U.S.D.A. Intermountain Forest and Range Experiment Station, Moscow, Idaho.

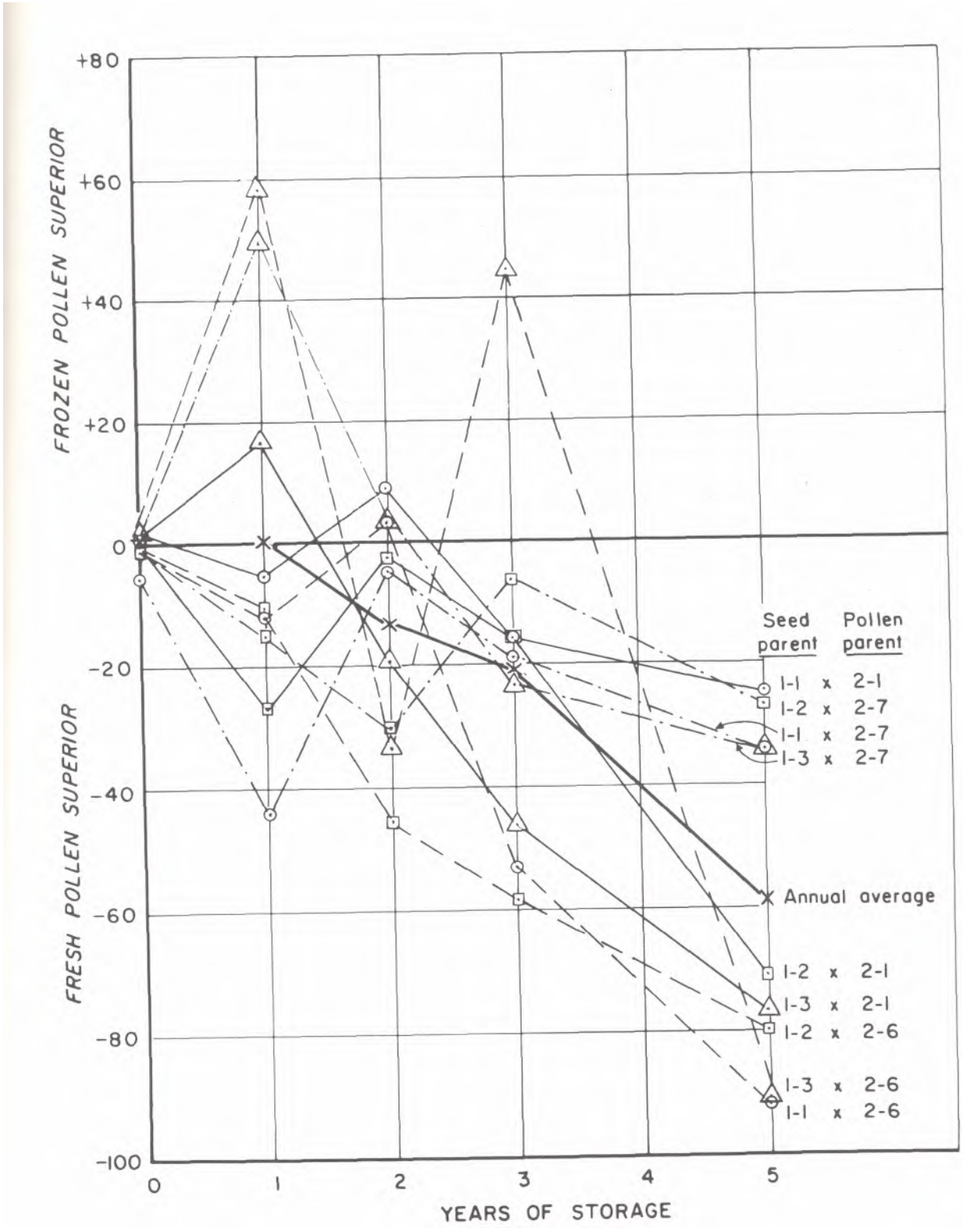

FIGURE 1. — Difference between percent of total seeds that were filled from frozen pollen and from fresh pollen for crosses on *Pinus monticola* during 5 years of pollen storage.

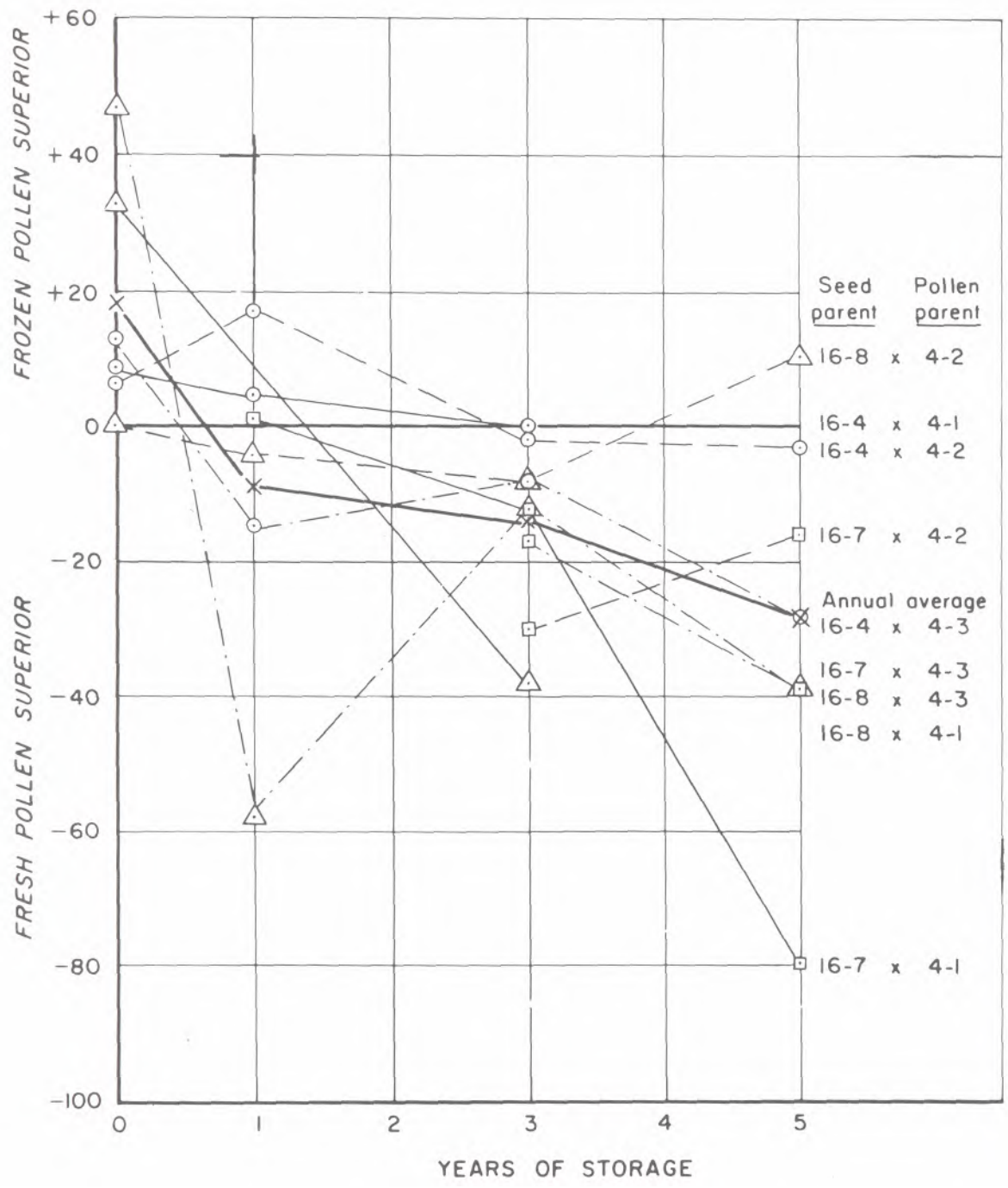

FIGURE 2. — Difference between percent of total seeds that were filled from frozen pollen and from fresh pollen for crosses on *Pinus jeffreyi* during 5 years of pollen storage.

higher yields of filled seeds than did frozen pollen. Frozen pollen also produced more total seed for *P. jeffreyi* and P. *monticola* but not for P. *sabiniana.* The increase was statistically significant only for P. *jeffreyi.* The indication that brief storage of pollen in a freezer increases seed yields requires further study for verification. Brief freezing apparently had a beneficial effect on pollen, but it may not be related to a change in pollen physiology.

Storing pollen in a freezer for 1 year had no significant influence on total seeds per cone or the proportion of the seeds that were filled. Data for three P. *monticola* seed trees showed that frozen pollen had a great advantage over fresh pollen on tree 1 - 3. This unique response of tree 1 - 3 to frozen pollens was not repeated in other years, and it cannot be explained. These results show that pollen frozen for 1 year does not lose its ability to set seed and to produce embryos. Probably all pine breeders should regularly freeze pollen that must be stored for a year before it is used.

The only species producing sufficient strobili for pollination in 1960 was *Pinus monticola.* Thus, data on effects of 2 years of storage are limited. The few cones that were produced on this species gave only 60 percent of normal seed yields. The frozen pollen produced fewer filled seeds per cone than fresh pollen, but the difference was not statistically significant. The proportion of all seeds that were filled following pollination with fresh pollen was about the same as in all other years. Thus, when few strobili are produced in some years, they can be expected to give normal proportions of sound seed but comparatively few sound seeds per cone. Breeding probably should be deferred when strobili are sparse.

Pollinations in 1961 with pollen frozen for 3 years produced significantly fewer seeds than pollinations with fresh pollen. This was true for both P. *monticola* and P. *jeffreyi.* Both total seeds per cone and proportion of seeds that were filled were less with frozen pollen. Crosses on two seed trees of *P. contorta* var. *murrayana* gave results that support these findings. Results of crosses on one seed tree of P. *ponderosa,* however, showed no deleterious effects of freezing pollen for 3 years.

Pollen frozen 5 years lost about half of its ability to produce sound seed. Frozen pollen produced about one-third as many filled seeds as fresh pollen for *P. monticola* and about two-thirds as many for *P. jeffreyi.* Differences between fresh and frozen pollen were statistically significant. Nevertheless, even after 5 years of storage some frozen pollens gave very high yields of filled seeds. These general conclusions are supported by the fragmentary data for the other three species.

On the average, frozen pollens declined gradually but steadily in ability to produce filled seeds after the first year (figs. 1 and 2). The initial increase in production of filled seeds after freezing pollen for a few days has already been mentioned. After 1 year of storage, frozen pollen produced about the same proportion of filled seed as did fresh pollen. The first significant decline occurred after 2 years of freezing. But even after 3 and 5 years of storage the pollens still would be useful in a breeding program. The remaining series of pollinations in this study will show how long frozen pine pollen retains its ability to produce sound seeds.

Pollen frozen 5 years did not lose its ability to set seeds. It kept ovules alive until seedcoats formed. Total number of seed in cones of *P. monticola* remained about the same after 1, 2, and 5 years of freezing, but in the third and fifth years frozen pollen gave fewer seeds per cone than fresh pollen. Crosses of *P. jeffreyi* that set cones did not show a significant reduction in total seeds. These results show that pollens frozen 5 years generally may be expected to set almost normal numbers of cones and seeds.

Pollen from certain trees was better than pollen from other trees after 5 years of freezing. The best pollen parent of *P. monticola* was tree 2 - 7. Pollen from this tree produced the most seeds per cone and the highest proportion of sound seeds. Pollens from trees 2 - 1 and 2 - 6 produced progressively lower proportions of sound seed. For *P. jeffreyi* pollen from tree 4 - 2 fertilized almost the same proportion of seeds after 5 years of freezing as at the beginning of the study. Pollen from tree 4 - 3 retained less of its ability to set sound seed. Pollen from tree 4 - 1, after performing normally in 1961, seemed to have lost nearly all of its viability in 1963. It set cones on two seed trees, but they failed to mature. It produced only one sound seed in five cones on the other seed tree. Perhaps the particular frozen aliquot used in 1963 was defective. Final conclusions on pollen from this tree must await the results of crosses after the next pollinations. Reasons for differences among trees in pollen viability are unknown. Maturity of the pollen when it was collected or its moisture content when placed into storage both might influence storage life. The data show that performance of a pollen after even 3 years of freezing cannot be used to predict its ability to produce seeds after 5 years.

Data for crosses using fresh pollen provide a unique opportunity to analyze compatibilities of seed and pollen parents during five different breeding seasons. The seed data for P. *monticola* were subjected to multiple graphical t tests. For each cross the mean plus or minus two standard errors of the mean was plotted (fig. 3). Crosses were considered significantly different if the plotted values did not overlap.

For seed tree 1- 1, pollen parents did not significantly influence number of filled seeds, total number of seeds, or proportion of filled seeds. The same was true for seed trees 1 - 2 and 1 - 3. Thus, pollen parents did not significantly influence seed production on any of the three seed trees. Seed parents did differ significantly in seed production. Generally the significant differences reflect the low total numbers of seeds set on tree 1 - 2. The

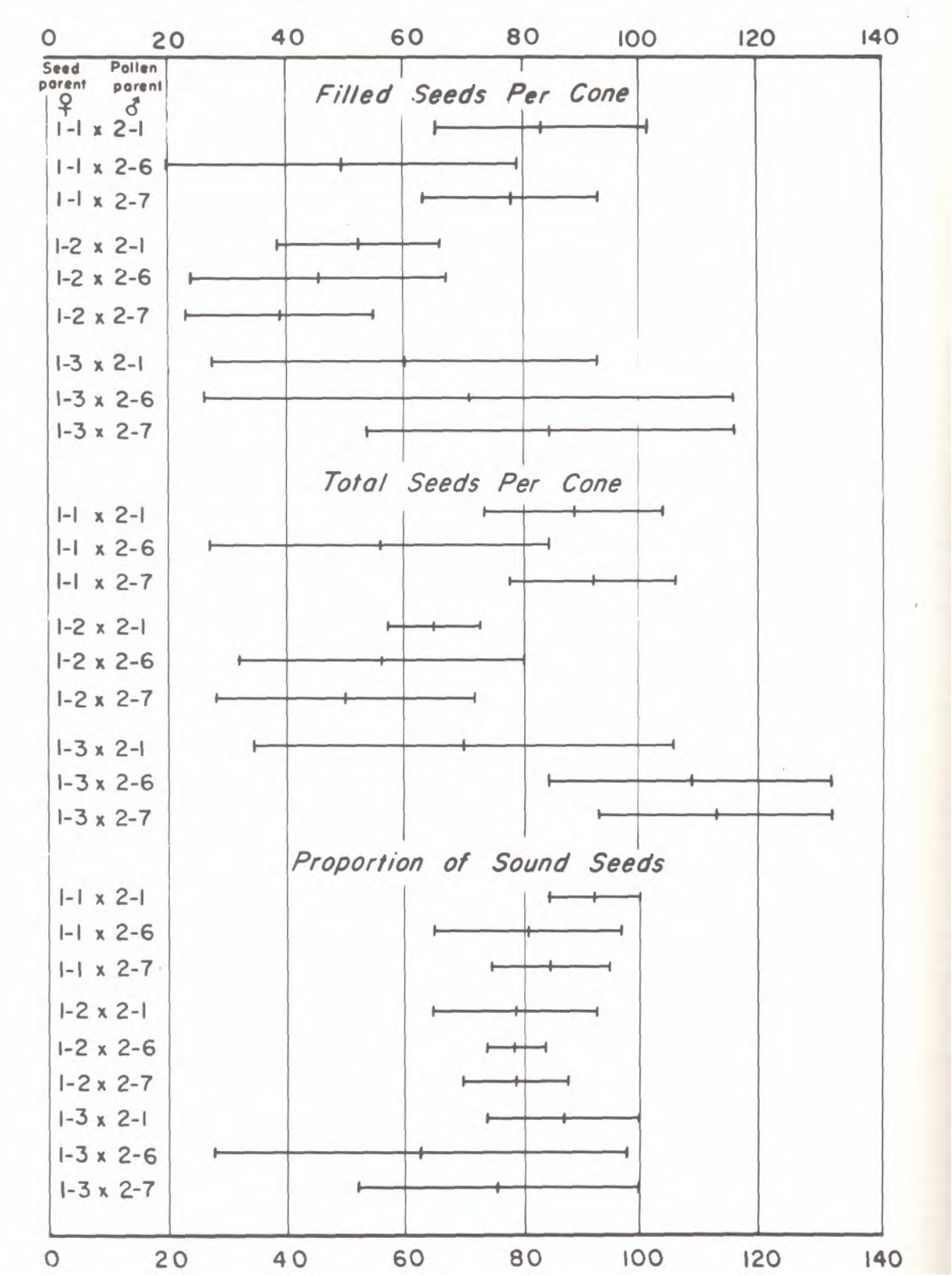

FIGURE 3. — Comparisons of means plus or minus two standard errors of the mean for seed produced by fresh pollen in nine crosses of *Pinus monticola* during 5 breeding seasons.

proportion of seeds that were filled was about the same for all crosses. Seed parents, pollen parents, or particular combinations of seed and pollen parents did not give significant differences. No significant incompatibilities were detected among the nine parental combinations.

Refrigerating pollen at 5°C. during the first 2 years of the test showed no advantages. After 1 year of storage it produced, on the average, less than 10 percent of the number of seeds produced by fresh or frozen pollen. Refrigerated pollen of *P. ponderosa* performed better than that of any other species. It yielded nearly half as many seeds as frozen pollen and one-third as many as fresh pollen. Pollen refrigerated for 2 years could be tested only on P. *monticola* in 1960, and it failed to produce any filled seed. From these results, storing pollen at 5°C. even for 1 year cannot be recommended.

These *in vivo* results show that caution should be used in interpreting the outcome of crossing experiments using year-old refrigerated pollens. Wright (1959) used year-old refrigerated pollen and had results from intraspecific and interspecific crosses similar to ours. Seed production following pollinations with year-old refrigerated pollen cannot be used to estimate genetic incompatibilities between species or seed production potentials from controlled crosses. Neither can seed production by several 1-year-old refrigerated pollens be compared because they differ in rate of deterioration in storage.

This study provided excellent data for comparing seed production from open and controlled pollinations (table 1). Average results from controlled crosses with three fresh pollens were compared with results from open pollination by wind. The

Table 1.--Total seeds and percent of seeds that were filled following controlled pollinations with fresh pollen (C) and open pollinations by wind (O)

Species	Seed tree	Pollen	Pollination year 1958		1959		1961		1963	
			Filled	Total	Filled	Total	Filled	Total	Filled	Total
			Percent	No.	Percent	No.	Percent	No.	Percent	No.
P. monticola	1-1	C	94	78	75	76	92	82	96	105
		O	76	40	75	193	--	--	--	--
	1-2	C	87	56	83	44	66	63	87	81
		O	73	33	76	84	--	--	--	--
	1-3	C	91	130	32	97	73	102	93	105
		O	79	68	94	82	65	83	--	--
P. jeffreyi	16-4	C	85	130	88	98	96	158	86	132
		O	91	124	94	138	--	--	--	--
	16-7	C	--	--	85	78	96	142	77	119
		O	--	--	69	86	--	--	--	--
	16-8	C	57	109	88	174	92	125	61	136
		O	69	127	73	151	92	72	--	--
P. contorta v. murrayana	13-1	C	--	--	38	32	71	23	83	18
		O	94	11	90	38	--	--	--	--
	13-2	C	--	--	84	17	94	22	92	28
		O	55	15	56	27	--	--	--	--
	13-3	C	70	8	74	9	89	6	75	23
		O	91	35	91	31	--	--	--	--
P. ponderosa	131-1	C	--	--	--	--	--	--	33	74
		O	--	--	--	--	--	--	--	--
	131-3	C	--	--	72	29	91	54	--	--
		O	--	--	84	19	--	--	--	--
	131-4	C	--	--	94	75	88	39	75	88
		O	--	--	91	90	74	31	--	--
P. sabiniana	12-1	C	64	96	96	86	--	--	89	40
		O	50	96	76	46	--	--	--	--
	12-2	C	--	--	--	--	--	--	81	96
		O	66	141	--	--	--	--	--	--
	12-3	C	70	95	99	31	--	--	32	82
		O	13	61	45	111	--	--	--	--

data are extremely variable, but 25 pairs of data were available for 5 species during 4 pollination years. Sign tests (Siegel 1956) showed that controlled and open pollinations did not differ significantly in total seed production or in proportion of seeds that were filled.

Pollen germination *in vitro* is reported here mainly to complete the story. Pollen germination tests were not designed for statistical comparisons of results. Tests were not conducted on the same day or even in the same month in some years, and provisions for replication and randomization in the tests were inadequate.

Fresh pollen collected in successive years had consistently high viability (table 2). After the second and third years of storage the frozen pollens showed germinability equal to that of fresh pollen; their decreasing ability to produce sound seeds has already been discussed. Refrigerated pollens, in agreement with *in vivo* results, showed a marked reduction in germinability after only 1 year of storage and very low germinability after

Table 2.--Percent of pollen grains germinating or bursting for fresh pollen collected each year and for pollen collected in 1958 and stored in a freezer at -20°C. or in a refrigerator at 5°C.

Species	Pollen parent	Pollen	Year of germination test				
			1958	1959	1960	1961	1963
P. monticola	2-1	Fresh	--	70	70	41	--
		Frozen	--	75	67	45	22
		Refrig.	--	60	32	30	--
	2-6	Fresh	--	50	50	74	--
		Frozen	--	77	77	77	52
		Refrig.	--	50	6	4	--
	2-7	Fresh	--	80	60	77	--
		Frozen	--	37	80	69	57
		Refrig.	--	40	4	4	--
P. jeffreyi	4-1	Fresh	60	0	70	75	70
		Frozen	--	39	65	55	22
		Refrig.	--	0	0	0	--
	4-2	Fresh	70	60	80	85	75
		Frozen	--	55	70	72	63
		Refrig.	--	30	0	10	--
	4-3	Fresh	90	0	90	49	90
		Frozen	--	0	60	19	35
		Refrig.	--	5	0	0	--
P. contorta v. murrayana	12-7	Fresh	50	--	90	70	80
		Frozen	--	54	77	47	38
		Refrig.	--	60	5	14	--
	12-8	Fresh	70	--	70	67	70
		Frozen	--	57	80	40	57
		Refrig.	--	50	9	9	--
	12-9	Fresh	60	--	80	60	78
		Frozen	--	30	59	34	60
		Refrig.	--	60	19	22	--
P. ponderosa	4-59	Fresh	80	70	70	26	80
		Frozen	--	65	87	70	45
		Refrig.	--	--	77	74	--
	4-61	Fresh	80	--	60	72	70
		Frozen	--	69	80	75	50
		Refrig.	--	--	67	60	--
	4-65	Fresh	90	80	80	80	10
		Frozen	--	64	90	69	45
		Refrig.	--	--	62	57	--
P. sabiniana	11-2	Fresh	80	80	80	70	88
		Frozen	--	44	64	55	50
		Refrig.	--	60	27	17	--
	11-3	Fresh	80	90	80	80	85
		Frozen	--	57	62	55	58
		Refrig.	--	70	52	18	--

2 and 3 years of storage. The exception to this story was pollen of P. *ponderosa.* Even after 3 years of refrigeration it germinated very well. Unfortunately it was not used in controlled pollinations, so its ability to produce sound seeds is unknown.

Normal cone and seed sets were produced by fresh and frozen pollens of P. *jeffreyi* that failed to germinate *in vitro* in 1959 (fig. 2). Other parallel results of seed production from pollen that failed to germinate *in vitro* are on record at Placerville.

The conclusion to be drawn from all of these findings is that pollen germinability *in vitro* indicates pollen viability, but only use of pollen *in vivo* will show its ability to set cones and to produce sound seeds. Germination tests *in vitro* probably have very limited value in predicting the ability of pine pollens to produce sound seeds.

Summary

The ability of pollens of five species of pines to set cones, to produce seed, and to yield filled seed after cold storage up to 5 years was determined. Pollen samples for each species were frozen at −20°C. for a few days and for 1, 2, 3, and 5 years. Two other samples for each species were refrigerated at about 5°C. for 1 and 2 years. Each pollen sample was tested for germination *in vitro* in the year in which it was used. Strobili on three seed trees of each species were pollinated with fresh and stored pollen from three other trees. Filled and hollow seeds from each cross were counted. Total seeds per cone and the proportion of seeds that were filled are reported.

Brief freezing of pollen for a few days between extraction and use significantly increased yield of filled seed. Storing pollen in a freezer for 1 year had no significant influence on seed yields. Pollen stored 2 years produced fewer filled seeds than fresh pollen. After 3 and 5 years of freezing, the reductions in yield of sound seeds were significant, becoming progressively greater as storage time increased. Results varied by species, but pollen frozen 5 years produced only about half as many filled seeds as fresh pollen.

However, freezing of pollen did not influence its ability to pollinate strobili and to permit seed coats to form. Brief freezing for a few days actually increased yield of seeds. Total yields of filled plus hollow seeds were about the same for pollen frozen for 5 years as for fresh pollen.

Pollens from different trees varied in ability to produce seeds after 5 years of storage. Pollen from one tree fertilized almost the same proportion of seeds after 5 years of freezing as at the beginning of the study. Another pollen seemed to have lost nearly all of its viability. Reasons for differences among pollens are unknown.

Pollens refrigerated at 5°C. for 1 year produced less than 10 percent of the number of seeds produced by fresh or frozen pollen. Species varied considerably in seed production after 1 year of storage. Pollen refrigerated for 2 years failed to produce any filled seeds.

Successive crosses using fresh pollens in five different breeding seasons failed to show any significant differences in seed setting ability among the pollen parents. Crosses in a year when few strobili were produced resulted in major reductions in seed yields. No genetic incompatibilities were apparent among the nine parental combinations produced each year.

No significant differences were found in seed production following controlled pollinations with fresh pollen and open pollination by wind. Controlled pollination did not result in more or less seed being produced.

Results of pollen germination tests *in vitro* are presented and discussed. The conclusion was drawn that results of such tests have little value in predicting the ability of pine pollens to produce sound seeds.

Literature Cited

Roden, R. W. 1958. Handling and storage of pollen in eucalyptus breeding. Austral. Forest. 22: 73-81.

Cumming, W. C. and F. I. Righter. 1948. Methods used to control pollination of pines in the Sierra Nevada of California. U. S. Dep. Agr. Circ. 792, 18 pp.

Duffield, J. W. and R. Z. Callaham. 1959. Deep-freezing pine pollen. Silvae Genet. 8: 22-24.

Ehrenberg, C. E. 1961. Studies on the longevity of stored pine pollen *(Pinus silvestris* L. and *I'. contorta* var. *murrayana* Engelm.) Meddel. Statens Skogsforskningsinst. 49 (7) .31 pp.

Righter, F. I. 1939. A simple method of making germination tests of pine pollen. J. Forest. 37: 574-576.

Siegel, S. 1956. Nonparametric statistics: for the behavioral sciences. McGraw-Hill, 312 pp.

Wright, J. W. 1959. Species hybridization in the white pines. Forest Sci. 5: 210-222.