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STORAGE OF SUGARBEET POLLEN[1]

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SUMMARY

To develop the technology for long-term pollen preservation, sugarbeet pollen was collected from plants grown in the greenhouse and in the field, and was stored 1 day to 1 year at 5, –18, and –196 °C. Pollen containing about 12% moisture was successfully stored in liquid nitrogen (LN_2) up to 1 year; this pollen effected fertilization of male-sterile flowers as well as freshly collected pollen. Germination of the resultant seed was good and not different from seed from fresh pollinations. Pollen stored at –18 °C for 1 year did not result in as much seed set as fresh pollen, and 1 year at 5 °C was essentially lethal. In vitro pollen germination served as a post-storage viability measure, provided the pollen was hydrated before germination. The methods tested in these experiments provided a relatively simple, reliable, and inexpensive means for preservation of sugarbeet pollen for breeding purposes and for preservation of genetic resources.

INTRODUCTION

A practical means of long-term storage of pollen is needed in research and breeding of many crops. Pollen storage facilitates procedures not otherwise possible, e.g., intermating of asynchronous flowering plants and the use of a unique pollen over an extended period. Long-term pollen storage also has potential for preserving plant genetic resources.

Various methods have been used to successfully store pollen of many species for varied lengths of time. Methods reported include vacuum and freeze-drying (KING, 1961; TOWILL, 1985), cool temperature and various means of refrigerated frozen storage (VISSER, 1955), liquid air (COLLINS et al., 1973), and liquid nitrogen (LN_2) (BARNABAS & RAJKI, 1976; TOWILL, 1985). In LN_2 at –196 °C, biological and chemical activities are totally inhibited (MAZUR, 1966), including those that normally result in cell damage or nutrient exhaustion. However, the physical effect of rapid freezing and thawing can be injurious to pollen, particularly to pollen with high moisture content (BARNABAS & RAJKI, 1976).

Although low temperature preservation of pollen from numerous species is reported, little information exists on the preservation of sugarbeet (*Beta vulgaris* L.)

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pollen. Since sugarbeet pollen is trinucleate (BREWBAKER, 1967) and, like most trinucleate pollen, is relatively short lived (ARSCHWAGER, 1940), long-term storage of sugarbeet pollen would be useful for breeding and research purposes as well as a means of preserving nuclear genetic stocks.

The purpose of our research was to investigate the feasibility of low temperature storage of sugarbeet pollen and to describe practical methods of collection and utilization.

MATERIALS AND METHODS

During the course of this research, sugarbeet pollen was collected from diploid flowering plants grown in the field, field-tent isolators, and greenhouses. A different heterogeneous population was used for each of the several pollen storage experiments. Although a vacuum pollen collection apparatus has been described (KROUCH et al., 1957), we found that collection of sufficient quantities of pollen was faster using a large glass plate or rigid, smooth-surfaced paper over which flowering plants were shaken. A flat blade was used to scrape the pollen from the plate onto a smooth paper which was then bowed, inclined, and tapped to remove the anthers and other debris. The relatively pure pollen remaining then was scraped into the collection vessel. Based on our previous experience (R. HECKER, 1985, unpublished), the pollen was handled as expeditiously as possible from collection to storage, germination, or fertilization. When pollen was desiccated, it was placed in a closed chamber containing anhydrous calcium chloride for 24 hours at 5 °C. For subsequent storage, the pollen was placed into small, plastic, leak-proof cryotubes with a screw cap and silicon gasket to maintain them airtight while in storage. Samples then were stored refrigerated at 5, −18 °C, or held in LN_2 (vapor phase or liquid, −150 to −196 °C). Storage periods of 1 to 365 days were used. All pollen subjected to storage was allowed to warm in cryotubes at 23 °C for 30 minutes, then without hydration was germinated in liquid medium at 23 °C (R. HECKER, 1985, unpublished) or blown onto open male-sterile (MS) flowers.

Post-desiccation moisture content of each pollen collection destined for storage was determined gravimetrically after oven drying samples at 103 °C for 72 hours. A differential thermal analysis (DTA) was conducted in some experiments to detect freezable water in the pollen (BURKE et al., 1976).

We tested pollen viability of each collection before storage and immediately following each storage treatment using in vitro germination (R. HECKER, 1985, unpublished) and pollination of open flowers on MS plants. After 24 hours of in vitro incubation in liquid medium at 23 °C, pollen germination counts were made directly in the petri dishes using a microscope at 100×. Remnant pollen was used to pollinate two or more MS plants, portions of which were bagged and the open flowers were counted at the time of pollination. Pollen was applied dry into the bag with an atomizer. The number of resultant seeds was counted. The seeds were then germinated in a standard germination test.

In those experiments involving pollen hydration, the pollen was held for 15 minutes at 23 °C in a small chamber lined with water saturated blotting paper.

Completely random designs with two or three replications were used for all experiments. For in vitro germination, at least 300 pollen grains were counted per replication.

All of the resultant seed from the pollinated MS plants were germinated (up to several hundred seeds per plant). Appropriate analyses of variance were done and LSD's were computed.

RESULTS AND DISCUSSION

An experiment to determine the moisture content of pollen and the presence or absence of freezable water in the pollen was conducted preliminary to the pollen storage experiments. Pollen collected from plants in the field contained 32% moisture. A positive DTA indicated the presence of freezable water. After desiccation for 24 hours, this same pollen contained 12% moisture, and the DTA was negative. The post-desiccation absence of freezable water indicated that dessicated pollen would escape injury during sub-freezing storage. Hence, all pollen destined for storage was desiccated.

Three experiments were conducted in which pollen collected from sugarbeet plants in the field was stored up to 1 year at 5, −18, and −196 °C (Table 1). In four more experiments, greenhouse-produced pollen was stored up to 175 days at −18 and −196 °C (Table 2). The pollen for each experiment was from a different population. Analyses of variance across experiments within the two studies showed no pollen source by treatment interaction. Variances within the two studies were homogeneous. All pollen was desiccated for 24 hours before storage. Moisture content of the field pollen in the three experiments ranged from 26 to 32% at collection, and 11 to 12% after desiccation. Greenhouse pollen had 16 to 21% moisture at collection, and 6 to 9% post-desiccation.

The results of three experiments (Table 1) indicate that pollen of 12% or less moisture

Table 1. In vitro germination at 23 °C of sugarbeet pollen from field plants, seed set (as % of open flowers at pollination), and germination of resultant seed; means of three experiments.

Pollen treatment	Pollen germination (%)	Seed set (%)	Seed germination (%)
None (control)	25	47	55
Desiccated	2	62	43
Desic., stored 24 hr			
5 °C	6	68	60
−18 °C	4	56	59
−196 °C	12	37	68
Desic., stored 26 days			
5 °C	7	13	60
−18 °C	5	14	49
−196 °C	5	21	71
Desic., stored 1 yr			
5 °C	0	2	68
−18 °C	2	19	37
−196 °C	6	37	65
LSD (0.05)	4	25	39

retained some viability and function after 1 year of storage at −18 °C, and in LN_2 at −196 °C. Pollen stored at 5 °C for 1 year lost most of its viability. The best measure of poststorage pollen quality in these experiments was the percentage of pollinated flowers that set seed. In vitro pollen germination without hydration of pollen was not an adequate viability measure. Later experiments demonstrated the necessity of hydrating desiccated pollen before it was was used for in vitro germination (R. HECKER, 1985, unpublished). However, desiccated pollen without hydration effected fertilization of MS flowers to the same degree as fresh untreated pollen, 62 vs. 47% seed set, respectively (Table 1). Storage of pollen desiccated for 24 hours at 5 and −18 °C appeared to have no deleterious effect on seed set, however, −196 °C may have caused a reduced seed set. Storage for 26 days or 1 year reduced the amount of seed set from pollen stored at 5 and −18 °C, but no further decline occurred from pollen stored at −196 °C, so that after 1 year, seed resulting from pollination of MS flowers germinated 37 to 71%, the control being 55%. Hence, the quality of seed resulting from fertilization with stored pollen was comparable to seed produced from fresh pollen.

Results of four similar experiments with greenhouse-produced pollen are in Table 2. The 5 °C storage treatment was deleted, based on the results of the previous experiments. As with field collected pollen, in vitro germination of nonhydrated pollen was not a good indicator of pollen viability of survival in storage. Seed set with greenhouse-produced pollen was not as good as with the field-produced pollen, but some of the pollen did survive post-desiccation storage at −18 and −196 °C in LN_2 for at least 175 days. The quality of the resultant seed was relatively good, with germination of 25 to 64%.

Because pollen moisture content at collection can vary considerably, it was necessary to determine the effect of LN_2 on pollen containing varied amounts of moisture. Two experiments to test the effect of LN_2 treatment of pollen having various moisture levels are summarized in Table 3. In experiment 1, pollen that had 17.7% moisture at time of collection was desiccated for up to 72 hours; the moisture was reduced to 5.4%. In experiment 2, pollen collected at 20.5% moisture was hydrated to different levels up to 36.0%. These experiments showed that LN_2 treatment of pollen commenced

Table 2. In vitro germination at 23 °C of sugarbeet pollen from greenhouse-grown plants, seed set (as % of open flowers at pollination), and germination of resultant seed; mean of four experiments.

Pollen treatment	Pollen germination (%)	Seed set (%)	Seed germination (%)
Desiccated 24 hrs (control)	2	24	48
Desic., stored 6 days			
−18 °C	4	19	64
−196 °C	3	16	46
Desic., stored 175 days			
−18 °C	4	7	25
−196 °C	1	9	63
LSD (0.05)	5	11	16

Table 3. Moisture and in vitro germination at 23 °C of sugarbeet pollen subjected to desiccation, LN_2 (24 to 52 hours), and pregermination hydration (15 min).

Pollen treatment	Moisture	Treatment and pollen germination			
		not frozen		LN_2	
		not hydrated	hydrated	not hydrated	hydrated
	(%)	(%)	(%)	(%)	(%)
Exp. 1					
Desiccated 72 hr	5.4	1	9	2	20
Desiccated 5 hr	7.7	7	19	4	18
Desiccated 2 hr	11.1	16	24	24	18
Fresh	17.7	26	27	16	20
LSD (0.05)	1.2	3.5	6.4	5.0	5.8
Exp. 2					
Fresh	20.5		13		–
Hydrated 5 min	20.8		6		2
Hydrated 10 min	20.4		14		1
Hydrated 15 min	22.7		12		1
Hydrated 20 min	26.3		11		0
Hydrated 40 min	26.6		10		0
Hydrated 60 min	–		11		0
Hydrated 75 min	36.0		13		0
LSD (0.05)	4.6		4.9		1.3

to be lethal between 18 and 20% moisture. This is apparently the moisture content above which the physical effect of rapid freezing and thawing is injurious to sugarbeet pollen. Similar high moisture freezing limits have been reported for seed of a number of species (BECWAR et al., 1983; STANWOOD, 1985).

The first experiment also demonstrated the beneficial effect of pregermination hydration (15 minutes) of desiccated pollen on in vitro germination. With 2 hours and 5 hours of desiccation, pollen germination significantly declined with unfrozen and LN_2 treatments, respectively, unless revived by hydration. The beneficial effect of hydration of dry pollen was not discovered until after the storage experiments had been completed. Hence, the pollen germination data in Tables 1 and 2 were from dessicated nonhydrated pollen, and likely were lower than they would have been with rehydration before germination.

Pollen in each of the several storage experiments was from different sources, all heterogeneous. There was no source by treatment interaction. We did not treat pollen from inbreds, hence, we do not know if pollen of specific genotypes might interact with storage treatment. This needs to be tested in order to detect nonrandom lethality over time. However, from our several different heterogeneous pollen sources, we have no evidence that indicates genetic differences for pollen storability.

Although all the pollen used in this study was from diploid plants, previous experiments (R. HECKER, 1985, unpublished) showed no difference in viability of pollen from diploid and autotetraploid plants. However, we have not compared the two pol-

lens after exposure to subfreezing temperature.

Although we did not test cryoprotectants, it is expected that there would be no cryoprotectant benefit for desicccated sugarbeet pollen, because there was little loss of viability of pollen in LN_2 for up to 1 year.

The production of viable seed in these experiments with pollen stored in LN_2 (−196 °C) or in a freezer (−18 °C) for up to 1 year demonstrated that pollen remained viable in frozen storage. Data for seed set in these experiments indicate that LN_2 may be the best storage environment for long periods. An experiment is currently in progress to test pollen storage in LN_2 for several years. Even if storage of pollen in LN_2 is not different than storage by refrigeration, LN_2 is likely to be more reliable for long-term storage.

Pollen preservation as a breeding tool could be adopted now for use in any situation where it would be necessary or desirable to hold pollen for future use. We have removed cryotubes from LN_2, taken some pollen, then replaced the tubes into LN_2, without apparent effect on the remnant pollen. However, if intermittent needs for pollen are anticipated, it may be advisable to divide the pollen into several cryotubes for LN_2 storage.

To simulate shipment of pollen before or after frozen storage, an experiment was conducted in which pollen from a heterogeneous source was germinated in vitro after collection (41% germination); after desiccation (42%); after desiccation and 1 day in LN_2 (37%); after desiccation and 5 days at 23 °C (34%); after desiccation, 1 day in LN_2, and 5 days at 23 °C (31%); and after desiccation, 5 days at 23 °C, and 1 day in LN_2 (36%). In each case, the pollen was hydrated prior to germination. Simulated shipment by holding pollen in an air tight cryovial for 5 days at 23 °C, before or after 1 day in LN_2, had no effect on pollen viability. The control (41% germination) was not significantly different than 5 days at 23 °C following 1 day in LN_2 (31%).

Frozen pollen can be thawed at room temperature in a few minutes, then hydrated. We have indirect evidence that pollen hydration does not improve the pollen's capacity to effect fertilization, but we have direct evidence that hydration is not deleterious (R. HECKER, 1985, unpublished data). However, for in vitro germination tests, it is essential that desiccated pollen by hydrated before germination.

Thawed, hydrated pollen can be dispensed dry onto flowering plants with an air bulb atomizing apparatus. The effective minimum quantity of pollen that can be dispensed is 4mg. Sugarbeet pollen weights about 310 mg/cc. Collection of as much as several grams of pollen is possible.

This study showed that sugarbeet pollen containing 12% moisture can be preserved in LN_2 or at −18 °C for at least 1 year, then used to effect fertilization. Pollen with up to 18% moisture was shown to survive the freezing and thawing process, but was not tested for fertilization ability. LN_2 treatment was essentially lethal to pollen that had 20% moisture or more.

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