Long-term Survival of Cryopreserved Sugarbeet Pollen

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ABSTRACT

Hecker and coworkers demonstrated that sugarbeet (*Beta vulgaris* L.) pollen could be stored in liquid nitrogen vapor phase (-160°C) (LN) for 1 year and remain viable. We demonstrate that pollen, stored for 17 years in LN was able to pollinate sugarbeet and produce viable seed. Two viability staining tests showed no significant differences between stored and fresh pollen, and differences in pollen tube germination were small. Long-term storage of pollen provides opportunities for uses in sugarbeet breeding and genetic resources preservation. In a heterozygous crop such as sugarbeet, collected pollen would be a way to preserve superior, individual genotypes for use over long periods of time. In plant breeding, stored pollen could be used as a long term tester (individual genotype or population) or from an individual genotype as the parent in a recurrent backcrossing scheme for genetic analysis. Collection and storage of pollen could be a way to obtain a more representative sample of the genetic diversity in wild populations. With restrictions on the international transport of seed becoming increasingly stringent, pollen could be an alternate way to distribute *Beta* germplasm. However routine use of *Beta* pollen collections requires a more complete understanding of physiological responses to LN storage.

Additional Key Words: *Beta vulgaris*, genetic resources, plant breeding, genetic diversity.

There has been a long collaboration between researchers at the USDA-ARS Sugarbeet Research Unit and the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) (formerly the National Seed Storage Laboratory). One of the projects has been the development of the technology necessary for long-term preservation of pollen (Hecker et al., 1986). Sugarbeet (*Beta vulgaris* L.) pollen collected in 1991 was desiccated (Connor and Towill, 1993) and then stored in LN. A sample of this pollen was retrieved from cryogenic storage in 2008 to test its viability and fertility.

Many uses for stored pollen in breeding programs have been discussed. These include the ability to pollinate asynchronously flowering plants, to store multiple genotypes in a small space, to easily ship genotypes, and to use an individual genotype over a long period of time (Barnabás and Kovács, 1997; Ganeshan et al. 2008; Hanna and Towill, 1995; Hecker et al., 1986). Long term pollen storage also offers interesting possibilities in the preservation and exchange of genetic resources. A small vial of pollen contains millions of haplotypes, which may provide a more representative sample of the genetic diversity in a population, may allow the reintroduction of genetic diversity into an existing in situ plant stand or field or greenhouse planted population, and could maintain genetic diversity in a species that requires a long generation time (e.g., tree species) (Ganeshan et al. 2008; Hanna and Towill, 1995; Hoekstra, 1995; Towill, 2004).

Hecker et al. (1986) demonstrated that desiccated sugarbeet pollen stored in liquid nitrogen vapor phase (-135°C to -160°C) (LN) was viable for 1 year. Some other crops for which pollen has been shown to be viable after long-term storage include corn (*Zea mays* L.) (Barnabás and Kovács, 1997), oil palm (*Elaeis guineensis* Jacq.) (Tandon et al., 2007), pear (*Pyrus* L.) (Reed et al., 1998), pecan [*Carya illinoinensis* (Wangenh.) C. Kockh] (Sparks and Yates, 2002), and nectarine [*Prunus persica* (L.) Batsch var. *nectarina* (Ait. f. Maxim.)] (Hanna and Towill, 1995). A number of other plant species, which had pollen that remained viable after being stored over 10 years, are listed in Ganeshan and Rajashekaran's review (2000) and Hanna and Towill (1995).

Pollen viability is tested using in vitro germination assays, vital staining and ability to produce progeny. Some of the most accurate assessments use vital stains such as fluorescein diacetate (FDA) and 3-(4,5-Dimethylthiazol-2-yl)-2-5diphenyl-2H-tetrazolium bromide (MTT) (Hoekstra, 1995). In sugarbeet, staining with FDA and MTT was related positively to pollen germination, with FDA results more consistent than MTT results (Hecker and McClintock, 1989).

MATERIALS AND METHODS

Pollen sources

Large quantities of sugarbeet pollen were collected on several days in late winter of 1991 from varied, heterozygous diploid sugarbeet varieties growing in the greenhouse. Pollen was dried to near optimal water content (Buitink et al., 1998) by placing it at room temperature over a saturated $MgCl^2$ solution [33% relative humidity (RH)]. Pollen was subsequently stored in liquid nitrogen vapor phase (-135°C to -160°C). Two vials were retrieved on different dates in April, 2008 for viability testing. Pollen, both fresh and stored, was allowed to hydrate on watch glasses in 100% RH in a closed germination box for 2 hr before viability assessments (Hecker and McClintock, 1988). Fresh pollen was collected on the same days that the vials were retrieved from cryogenic storage to serve as positive controls. The fresh pollen was cleaned and processed (Hecker and McClintock, 1988). Water content of pollen was measured using an 8-10 mg sample of fresh or 10-34 mg of stored pollen. Dry mass was measured after heating samples at 100°C for 24 hr. Differences between the stored and fresh pollen were analyzed using a two-sample Student's t-Test assuming unequal variances (Microsoft Excel - Data Analysis).

Pollen vital staining

Pollen viability was compared with vital staining techniques using FDA (fluorescein diacetate) and MTT (tetrazolium bromide) (Hecker and McClintock, 1989). Both were easily tested in the small sample available to us. One sample was taken from the first vial opened, and three samples from the second vial. Fresh pollen was collected on each of the days the vials were opened as described above. The percentage of positive staining after 30 min incubation in FDA (0.01%) (Hecker and McClintock (1989) adapted from Heslop-Harrison et al. (1984)) was evaluated on at least 300 pollen grains. The percentage of positive staining after 30 min incubation in MTT (0.5%) (Hecker and McClintock, 1989) was evaluated on at least 300 pollen grains. Differences between the stored and fresh pollen were analyzed using a two-sample Student's t-Test assuming unequal variances (Microsoft Excel - Data Analysis).

Pollen germination testing

Percentage of in vitro germination of 3 subsamples from one of the vials of stored pollen and 3 subsamples of fresh pollen was compared after 24 hr of incubation on pollen germination medium at

24°C (Hecker and McClintock, 1988). At least 300 pollen grains were counted for each subsample of the germination testing.

Seed set determination

Fertility of stored pollen was measured from seed set following pollination of 3 flowering annual, male sterile sugarbeet plants of FC404CMS (Panella and Hecker, 1995) or SLC03CMS (Owen, 1950). A small amount of pollen (20 mg plant^{-1}) was used in the pollination assay because of the limited supply of fresh pollen. Fresh or stored pollen was brushed on to the flowers using a paint brush, and then the plants were bagged for 24 hr to prevent airborne cross contamination. Plants were placed in a greenhouse chamber isolated from other pollen sources and allowed to set seed.

Seed from individual plants was harvested after 2 months and weighed. Seed from plants pollinated by the 20 mg aliquot was planted in the greenhouse, and the number of surviving seedlings per gram of seed determined. About 1400 mg of pollen was available from the stored sample and this was used to pollinate additional plants in a separate chamber.

RESULTS AND DISCUSSION

In this study we tested pollen that had been stored for 17 years in LN. There were differences in the moisture contents of the stored (12.6% of fresh weight) and fresh (25.6% of fresh weight) pollen (Table

Table 1. Viability measures of stored and freshly collected pollen. Pollen from 2 vials that were stored in separate locations within the same cryovat was used. Fresh pollen was collected from plants flowering in the greenhouse on the day the vials were opened. In each case, at least 300 pollen grains were counted for each sample.

		Pollen Staining Percent fluorescing	
Pollen	**Percent Moisture**[†]	**MTT**[‡]	**FDA**[§]
Stored	12.65	56.6	82.0
Fresh	25.59	85.6	91.8
$P < 0.05$	**	NS	NS

[†] Percent moisture (of fresh weight) was calculated from the stored pollen as it was removed from the vial. Fresh and stored pollen was rehydrated before the viability staining procedures.

[‡] tetrazolium bromide

[§] fluorescein diacetate

1) because the pollen was dehumidified before storage. After the pollen was allowed to hydrate at 100% relative humidity for 2 hr, it was tested for viability. The two staining methods used in this study to test viability of pollen showed no significant differences between the stored pollen and fresh pollen (Table 1). There was a larger variance in the stored pollen measurements (data not shown). In vitro pollen tube germination was measured in three subsamples from the second vial opened and compared with fresh pollen. Maximum germination values of approximately 26% were obtained from field collected pollen (Hecker et al., 1986). Germination of 17 year old and fresh pollen in this study was 10-11% (Table 2), which was higher than the 4% measured after 1 year of storage in LN reported by Hecker et al. (1986).

The definitive test of pollen viability is its ability to successfully fertilize flowers to form viable seeds. In comparison with fresh pollen,

Table 2. Percent germination of stored and fresh sugarbeet pollen. Three subsamples of rehydrated pollen from the second vial were tested for pollen tube germination and compared with 3 subsamples of hydrated fresh pollen, collected from plants flowering in the greenhouse on the day the vial was opened. At least 300 pollen grains were counted for each subsample.

Pollen	Percent Germination	Standard Deviation
Stored	10.37	4.04
Fresh	11.90	2.00

Table 3. Comparison of seed yield, number of seedlings, and seedling per gram of seed between plants pollinated with stored and fresh pollen. Twenty milligrams of rehydrated, stored pollen from the second vial were used to pollinate 3 flowering annual, male sterile sugarbeet plants of FC404CMS or SLC03CMS, and seed set was compared with hydrated fresh pollen, collected from plants flowering in the greenhouse on the day the vial was opened.

Pollen		Seed weight, g	Number of seedlings	Seedlings per gram
Stored	Mean	0.12	28.33	237.88
	SD†	0.08	16.92	54.23
Fresh	Mean	0.26	69.00	191.30
	SD	0.20	61.73	166.49‡

† Standard deviations of 3 subsamples.

‡ Although a seed weight was recorded, one of the pollinations produced no seedlings causing a high standard deviation.

the stored pollen successfully fertilized two male-sterile lines; however, seed yield from these plants was lower than that obtained from plants fertilized with fresh pollen (Table 3). All flowers were fertilized with an abundance of pollen (stored or fresh), and the lower seed yield from the stored pollen may be due to a reduced fertility in stored pollen. If this is the case, it would be important to know if the reduction in fertility is random or if there is selection on specific pollen haplotypes, because this could cause altered gene frequencies in plants produced from the stored pollen, compared to the original pollen donor population. A larger amount of pollen (approximately 200 mg plant^{-1}) yielded an average of 0.19 g seed plant^{-1} in 7 plants pollinated with excess stored pollen (data not shown).

Hecker et al. (1986) demonstrated that sugarbeet pollen could be stored in LN for 1 year and remained viable for another 5 days, which allowed shipment to other locations for use (Hecker et al. 1986). We have shown that sugarbeet pollen remains viable for at least 17 years when stored in LN. In a heterozygous crop such as sugarbeet, stored pollen could preserve superior, individual genotypes developed in plant breeding programs. For example, stored pollen from a superior tester (or small tester population) could be used for test crossing for combining ability, without needing to grow, vernalize, and synchronize the tester's flowering with the genotypes to be tested. The pollen could be stored for years, allowing comparison to a single genotype over time. When developing populations for genetic analyses that require backcrossing (e.g., inbred backcross line method) (Wehrhahn and Allard, 1965), or to obtain recurrent backcross populations, an individual rather than a population could be used as the recurrent parent.

Collecting wild relatives of cultivated plants provides challenges when there is insufficient seed to fully represent the total genetic diversity present. Collection of pollen could be a way to preserve a more representative sample of the genetic diversity present in wild populations. It could be used to pollinate increases within that population in the future, or to pollinate a male-sterile domesticated population to retain a larger sample of the variation from the wild population during introgression into a cultivated crop plant background. And finally, with restrictions on the international transport of seed increasingly stringent, pollen could be an alternate way to distribute genetic resources of cultivated beet germplasm internationally.

We have shown that pollen from heterozygous diploid sugarbeet varieties can undergo long term LN storage and remain viable. Widespread and routine use of *Beta* pollen collections, however, requires a better understanding of genotype specific responses to LN

storage, especially from lower vigor inbred lines. This report provides no information on how the effects of changes in collection, handling, or storage of sugarbeet pollen would impact viability. Also, there is no information on how pollen of other taxa within the Genus *Beta* would react to long-term LN storage. Nonetheless, the viability of these two cultivated sugarbeet pollen samples after 17 years suggests that successful long-term storage is a possibility.

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