

# Chapter 25: Collecting pollen for genetic resources conservation

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## Abstract

Pollen is a useful source of diverse alleles within a genepool and so may be an effective propagule for genebanks. The ease of pollen storage and shipment and the potential for its immediate use provide researchers with increased options when designing their breeding programs. Methods for pollen collection, desiccation, viability testing and longevity assessment have been developed for many species of interest, and have revealed the critical importance for increased longevity by using high quality pollen desiccating it sufficiently in a rapid manner and subsequently storing it at very low temperatures. Reliable viability assessments are dependent upon adequate rehydration and the use of reliable stains, *in vitro* germination assays or *in vivo* pollination experiments. Pollen preservation in genebanks will likely be implemented as standard procedures for handling and assessing it are developed.

## Current Status

### *Advantages to the use of pollen*

- Genebanked pollen can be made available to breeders upon request. For tree species, this obviates the need for growing the male parents in the breeding orchards. It allows for wide hybridization across seasonal and geographical limitations, and reduces the coordination required to synchronize flowering and pollen availability for use in crosses (Bajaj 1987). With adequate pollen available, one can also load additional pollen onto stigmas to increase pollination and yield.
- Pollen is available for research programs. As single cells, pollen provides a simple model system for research on conservation. Storage of pollen within genebanks also ensures its availability year-round for basic biology and allergy research programs (Shivanna 2003).
- Pollen captures diversity within small sample sizes, and documentation is available for long-term survival of pollen from many diverse species (table 25.1). Pollen also serves as a source of genetic diversity in collections where it is hard to maintain diversity with seeds (species of low fecundity, large seeds, or seeds that require an investment of labour to store).
- Pollen can also be shipped internationally, often without threat of disease transfer (Hoekstra 1995).

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This chapter is a synthesis of new knowledge, procedures, best practices and references for collecting plant diversity since the publication of the 1995 volume *Collecting Plant Genetic Diversity: Technical Guidelines*, edited by Luigi Guarino, V. Ramanatha Rao and Robert Reid, and published by CAB International on behalf of the International Plant Genetic Resources Institute (IPGRI) (now Bioversity International), the Food and Agriculture Organization of the United Nations (FAO), the World Conservation Union (IUCN) and the United Nations Environment Programme (UNEP). The original text for Chapter 25: Collecting Pollen for Genetic Resources Conservation, authored by F. A. Hoekstra, has been made available [online](#) courtesy of CABI. The 2011 update of the Technical Guidelines, edited by L. Guarino, V. Ramanatha Rao and E. Goldberg, has been made available courtesy of Bioversity International.

Table 25.1. A Selection of Species for Which Pollen Can Be Successfully Stored at –80°C or Liquid Nitrogen (LN) Temperatures

Species	Storage duration	Temperature	Viability test	Reference
<i>Actinidia</i>	1 y	LN	<i>in vitro</i> germ, pollination	Abreu and Oliveira 2004
<i>Aechmea</i>	15 min	LN	<i>in vitro</i> germ	Parton et al. 2002; Parton et al. 1998
<i>Allium</i>	1 y	LN	pollination	Ganeshan 1986b
<i>Beta</i>	17 y	LN	FDA, MTT, pollination	Panella et al. 2009
<i>Beta</i>	1 y	LN	pollination	Hecker et al. 1986
<i>Carica</i>	485 d	LN	<i>in vitro</i> germ, pollination	Ganeshan 1986a
<i>Carya</i>	13 y	LN	<i>in vitro</i> germ	Sparks and Yates 2002
<i>Carya</i>	1 y	LN	pollination	Yates and Sparks 1990
<i>Carya</i>	3 y	-80	<i>in vitro</i> germ, pollination	Yates and Sparks 1990
<i>Citrus</i>	3.5 y	LN	<i>in vitro</i> germ, pollination	Ganeshan and Alexander 1991
<i>Clanthus</i>	3 h	LN	<i>in vitro</i> germ	Hughes et al. 1991.
<i>Dioscorea</i>	2 y	-80	acetocarmine	Ng and Daniel 2000
<i>Elaeis</i>	8 y	LN	FDA, <i>in vitro</i> germ	Tandon et al. 2007
<i>Gladiolus</i>	10 y	LN	<i>in vitro</i> germ, pollination	Rajasekharan et al. 1994
<i>Glycine</i>	7 d	LN	pollination	Tyagi and Hymowitz 2003
<i>Guzmania</i>	15 min	LN	<i>in vitro</i> germ	Parton et al. 2002
<i>Humulus</i>	2 y	LN	pollination	Haunold and Stanwood 1985
<i>Juglans</i>	2 y	LN	<i>in vitro</i> germ	Farmer and Barnett 1974
<i>Juglans</i>	1 y	LN	<i>in vitro</i> germ, pollination	Luza and Polito 1987
<i>Lycopersicon</i>	5 wk	-80	pollination	Sacks and St. Clair 1996
<i>Lycopersicon</i>	22 mo	LN	<i>in vitro</i> germ, pollination	Karipidis et al. 2007
<i>Olea</i>	1 h	LN	<i>in vitro</i> germ	Parfitt and Almehti 1984a
<i>Panax</i>	11 mo	LN	stain, <i>in vitro</i> germ, pollination	Zhang et al. 1993
<i>Persea</i>	1 y	LN	pollination	Sedgley 1981
<i>Phoenix</i>	435 d	LN	<i>in vitro</i> germ, pollination	Tisserat et al 1983
<i>Protea</i>	1 y	LN	<i>in vitro</i> germ, pollination	Van der Walt and Littlejohn 1996
<i>Prunus</i>	12 mo	-80	<i>in vitro</i> germ	Martinez-Gómez et al. 2002
<i>Prunus</i>	1 h	LN	<i>in vitro</i> germ	Parfitt and Almehti 1984b
<i>Pyrus</i>	3 y	LN	pollination	Akihama and Omura 1986
<i>Rosa</i>	8 wk	LN	<i>in vitro</i> germ	Marchant et al. 1993
<i>Rosa</i>	1 y	LN	hanging drop, fertilization	Rajasekharan and Ganeshan 1994
<i>Solanum</i>	10 min	LN	<i>in vitro</i> germ	Towill 1981
<i>Tillandsia</i>	15 min	LN	<i>in vitro</i> germ	Parton et al. 2002
<i>Vitis</i>	64 wk	LN	<i>in vitro</i> germ	Ganeshan 1985a
<i>Vitis</i>	5 y	LN	<i>in vitro</i> germ, pollination	Ganeshan and Alexander 1990
<i>Vitis</i>	1 h	LN	<i>in vitro</i> germ	Parfitt and Almehti 1983
<i>Vriesea</i>	15 min	LN	<i>in vitro</i> germ	Parton et al. 2002
<i>Zea</i>	120 d	LN	<i>in vitro</i> germ, pollination	Barnabás and Rajki 1976

***Disadvantages to the use of pollen***

- Limited pollen production in some species. The primary limitation in the routine implementation of pollen storage within genebanks is the difficulty in obtaining adequate quantities of pollen for many species.
- Labour-intensive collection or processing. For some species, pollen is readily available, but resources to accumulate and process enough pollen for routine storage and distribution are inadequate.
- No standardized processing or viability-testing protocols. Processing and viability-testing methods have not been documented and standardized in a manner similar to that of seed testing.
- Regeneration of aged pollen. Seed regeneration can often be performed directly using the seed samples in storage. For pollen, associated mother plants are necessary to replenish pollen supplies when quantities are depleted or have deteriorated (Schoenike and Bey 1981).

***Pollen collection***

Collected pollen serves to maintain and preserve the alleles of an individual or population. Sampling strategies have often recommended collecting a set number of individuals per population to ensure that the common alleles are captured. The exact number of individuals that most effectively captures the genetic variation is dependent upon the genetic diversity and life-history traits of the species (Lockwood et al. 2006). Namkoong (1981) suggests that collecting pollen from a single tree easily captures the alleles for that genotype; however, it is recommended that a minimum of 68 trees be sampled to represent a wild population. Pollen can also be collected from individual trees within a genebank both to conserve alleles specific to each individual and to provide male gametes for breeding purposes. Although only small quantities of pollen are required to capture the genes of an individual, because of the challenges of pollen collection and processing, it might be more efficient to collect larger quantities to ensure its long-term availability to the user community.

Pollen should be harvested soon after anthesis, usually in the morning hours (Ganeshan et al. 2008; Towill 2004). Shelf life is short for pollen collected from immature, aged, or weather-damaged anthers (Towill 1985). It is usually more practical to collect anthers in the field and then separate the pollen grains from the anthers in a laboratory environment soon after collection. All pollen must be processed immediately (within hours) to ensure maximum potential longevity.

***Pollen desiccation***

Successful pollen genebanking is dependent upon achieving long-term survival of stored pollen. Water content, cooling rate and storage temperature all affect the longevity of stored pollen (Buitink et al. 1996, 2000). Field conditions and relative humidity at the time of harvest affect the pollen moisture content, and germinability is impaired when pollen is kept for any length of time in wet or high-humidity conditions (Hoekstra 1986). Pollen ages quickly when held at 24°C and 75% relative humidity (RH) (Van Bilsen et al. 1994).

For desiccation-tolerant pollen, it is critical that the pollen be dried to a target moisture content soon after harvest. Depending on species, successful long-term storage requires that the moisture content be reduced to or below levels at which there is no free water (Priestley 1986). For many species, pollen can be dried to water contents of 0.05 g H<sub>2</sub>O g<sup>-1</sup> dry weight (DW) without a loss in viability (Hoekstra 1986). This can be achieved by drying overnight in a low-humidity room environment or over salt chambers that are maintained at RH of about 30%. Equilibration over salt slurries, such as magnesium chloride or calcium nitrate, prevents damage that could result from over drying within ovens. It is a straightforward method to control moisture content in diverse laboratory environments (Connor and Towill 1993; Towill 1985).

Anthers or pollen grains can also be dried over silica gel at room temperature (Ganeshan 1985; Parfitt and Ganeshan 1989; Parton et al. 1998; Sacks and St. Clair 1996; Van der Walt and Littlejohn 1996). Martínez-Gómez et al. (2002) successfully desiccated almond pollen with silica gel for 48 hours at 22°C for long-term storage. Sato et al. (1998) dehydrated anthers at 20°C for 16–24 hours at RH of 15% or 32% prior to storage. Although some researchers have demonstrated successful desiccation through the use of freeze-driers for pollen desiccation, concerns have arisen with regard to maintaining viability in pollen that has

been frozen prior to dehydration (Ganeshan and Alexander 1986, 1987; Perveen and Khan 2008). Towill (1985) argued that vacuum drying was as effective as freeze-drying for maintaining pollen viability. Although air at low RH will increase the drying rate, pollen must be removed before it dries to a lethal moisture level (1% to 2% for peach and pine, 3.5% for coconut pollen) (Towill 1985). Pollen can be successfully dried in 35°C ovens, but care must be taken not to over dry under these conditions (Yates et al. 1991).

Rapid air-drying can also be achieved by using specialized pollen-dryers that blow air at 20% to 40% RH and 20°C, to quickly reduce moisture content in the pollen of *Poaceae* species, including *Avena*, *Pennisetum*, *Saccharum*, *Secale*, *Triticum*, *Tricosecale* and *Zea* (Barnabás and Kovacs 1996). Maize pollen is easily stored when quickly dried to 0.19 g H<sub>2</sub>O g<sup>-1</sup> DW (Buitink et al. 1996). The longevity of the pollen from these desiccation-sensitive species, and its tolerance to freezing temperatures, has been extended as a result of using rapid dehydration methods. The principle of rapid drying (flash drying) has successfully been documented in recalcitrant seeds, where it was shown that one could dry to a much lower water content if one did it rapidly (Pammenter et al. 1991).

### **Storage temperature**

It is possible to store pollen of many species at temperatures between 4°C and –20°C for the short-term. Dry pollen that is kept at between 4°C and –20°C remains viable for a few days to a year, which may be adequate for use in breeding programs (Hanna and Towill 1995).

Long-term viability can be maintained by storing pollen at –80°C or LN temperatures (–196°C) (Hanna and Towill 1995). Once desiccated, pollen can be dispensed into cryovials for long-term storage in LN or LN vapour. Precise labelling of vials and storage locations is recommended to aid in future retrieval of samples. Vials can then be placed in boxes or cryocanes and directly immersed in the liquid or vapour phase of liquid nitrogen (Barnabás and Kovács 1996; Ganeshan et al. 2008; Hanna and Towill 1995; Connor and Towill 1993).

### **Pollen rehydration**

Dried pollen is susceptible to injury from rapid water uptake during rehydration (also known as *imbibitional injury*) (Hoekstra and Van der Wal 1988), which can severely reduce germination and lead to low viability counts if vital staining (stains to identify living cells) is used to assess it. Low temperatures can exacerbate imbibitional damage, which is believed to arise from mechanical damage to the plasma lemma as polar lipids undergo phase changes as a result of fluctuations in temperature, water content and sugars (Hoekstra et al. 1992; Hoekstra and Van der Wal 1988; Crowe et al. 1989). Slow rehydration ameliorates imbibitional damage to pollen grains and this is usually accomplished by placing the pollen in a humid environment prior to direct liquid exposure (Hoekstra and Van der Wal 1988; Luza and Polito 1987; Parton et al. 2002). Pollen rehydration can be as straightforward as placing open vials of pollen in 100% humidity environments for 1 to 4 hours at room temperature (Connor and Towill 1993; Hanna and Towill 1995).

Although suboptimal storage conditions may affect pollen vigour before a measurable change in pollen viability is observed, most studies make use of viability assessments (Shivanna et al. 1991). Pollen viability can be measured by vital staining pollen grains, by germinating pollen grains *in vitro*, or by demonstrating successful fertilization and seed development in plants.

### **Pollen viability**

#### **Staining**

One commonly used vital stain is the fluoregenic ester, fluorescein diacetate (FDA). This test measures membrane integrity. Pollen grains fluoresce green when a cellular esterase cleaves the FDA (Heslop-Harrison and Heslop-Harrison 1970). Since this assay is dependent upon functional membranes, the osmoticum of the FDA staining solution is critical; stain is often dissolved in a 10% to 20% sucrose solution containing boric acid and calcium nitrate to minimize plasmolysis and membrane leakage.

Comparisons of viability determined through the use of FDA or tetrazolium and those obtained using *in vitro* germination or *in vivo* fertilization tests reveal consistently high correlations, provided pollen is adequately rehydrated prior to testing (Firmage and Dafni 2001; Khatun and Flowers 1995; Rodriguez-Riano and Dafni 2000; Shivanna and Heslop-Harrison 1981). FDA has occasionally been shown to give false negative results, where viable pollen appears dead (Heslop-Harrison et al. 1984).

Several tetrazolium-based stains are available for testing pollen viability (Norton 1966). The 3(4,5-dimethyl thiazolyl 1-2)2,5-diphenyl tetrazolium bromide (MTT) test was shown to give the most dependable results in a comparison trial using plum pollen (Norton 1966). In general, tetrazolium tests measure the ability to reduce colourless tetrazolium to coloured formazan, thus identifying pollen that has a capacity for oxidative metabolism (Hauser and Morrison 1964).

Many other vital stains have been developed and proposed over the past 50 years. Stains such as Alexander's, acetocarmine, aniline blue and X-gal have been shown to be successful identifiers of viability for relatively few species or under specialized conditions (Rodriguez-Riano and Dafni 2000). Viability results obtained with these stains may not correlate well with *in vitro* germination assays (Towill 1985).

### *In vitro* germination

Pollen can be germinated *in vitro* by placing pollen grains onto a medium and measuring the elongation of the pollen tube after a few hours of imbibition. Pollen tubes that elongate to a length that is at least the diameter of the pollen grain are considered viable (Dafni and Firmage 2000). Automated counting procedures using morphometry software result in pollen counts that are within 5% of visual observations and allow the determination of pollen tube length in addition to the data obtained by eye on tube presence or absence (Pline et al. 2002). These automated systems may expedite time-consuming assays of *in vitro* pollen germination. As for viability testing, it is important to implement repeatable and standardized methods and to use dead pollen samples as controls.

The optimal temperature for *in vitro* germination assays can be species dependent. The pollen from many species germinates well at 25°C; however, differences exist. For example, cotton pollen has an optimum germination temperature of 28°C to 31°C (Burke et al. 2004). Hence, for the purpose of pollen conservation, such information should be known for the target species.

*In vitro* germination methods utilize pollen immersed in aerated solutions, "hanging" drops, or dispersed on solidified medium. The medium is often that described by Brewbacker and Kwack (1963) or a slight modification thereof. Boric acid, calcium nitrate and sucrose concentrations in the medium might have to be optimized according to species (Bolat and Pirlak 1999; Heslop-Harrison 1992). The hanging-drop method involves the placement of a slide or coverslip with liquid medium and pollen inverted over a 100% humidity chamber (Rajasekharan and Ganeshan 1994). For observation, the slide is returned to an upright position and observed under a microscope.

### Pollination

Testing viability by observing pollen tube elongation within the stigma or fertilization and subsequent seed production is the most time-intensive way to determine pollen viability; however, these kinds of tests are also the most relevant to demonstrate the adequacy of the pollen for use. Rehydrated pollen can be placed on the stigmas of live plants, and tube length is measured after a pre-determined time interval (Dafni and Firmage 2000). Ideally, successful fruit set and seed production occurs after pollination with conserved pollen. Marquard (1992) demonstrated that high levels of viability are not required. Fruit set occurred when stigmas were treated with only 5% viable pollen. When pollen appears viable based on germination tests, it is often also viable in fertilization assays.

### **Pollen longevity**

The longevity of pollen is dependent upon many factors specific to cultivars or species as well as handling procedures, as discussed here (Ganeshan and Alexander 1991; Hanna and Towill 1995). The presence of sucrose and polysaccharides in the pollen has been correlated with protection of membranes from

desiccation or temperature stress and may confer greater longevity (Dafni and Firmage 2000; Hoekstra et al. 1989). High-quality pollen dehydrated to an optimal moisture content and stored at LN temperatures has been documented to store for well over 10 years (Panella et al. 2009; Sparks and Yates 2002). The low temperature reduces the molecular mobility in the cytoplasm, which may be a controlling factor in pollen longevity (Buitink et al. 2000). The aging of dried pollen is likely caused by oxidative reactions, and pollens with higher levels of unsaturated fatty acids usually have a shorter shelf life (Hoekstra 2005). Correspondingly, pollen longevity may be further improved by storing desiccated pollen in an oxygen-free atmosphere (Hoekstra 1992).

Most reports describing pollen survival after LN exposure state viability levels after determined lengths of time, often without initial germination data. These end-point levels serve to demonstrate that the tested length of storage is possible, but they do not describe the longevity of the pollen per se. Table 25.1 demonstrates the diverse range of species for which pollen can be placed at LN temperatures. According to current information, it is clear that both desiccation-tolerant and non-desiccation-tolerant pollen types can be stored for over 10 years under controlled conditions (Barnabás 1994; Barnabás and Kovács 1996; Shivanna 2003). Thus, despite additional challenges that may be present in storing desiccation-sensitive pollen, it is possible. Additional research is needed to determine how long both types of pollen will remain viable under these conditions.

## Future challenges/needs/gaps

Technologies for successful pollen conservation have been developed and are available. A set of standardized methods to process pollen types with different physiologies is needed to make pollen storage a routine effort in genebanks. For many species in need of pollen conservation, we need to know more about the phenology of pollen production so that we can properly time pollen harvests. Standards should be developed for pollen collection, processing and storage of desiccation-tolerant and non-desiccation-tolerant pollen types. Determination of pollen genebanking standards is an initial step towards implementing pollen genebanking methods.

The literature currently lists the age and viability of pollen from many species stored at LN temperatures (table 25.1) (Barnabás and Kovács 1996; Ganeshan and Rajashekaran 2000; Hanna and Towill 1995; Towill 1985). However, the viability over time, or longevity, of pollen stored under LN genebanking conditions has not been thoroughly evaluated. In addition, detailed biophysical studies should be pursued to determine the optimal water content, desiccation rates and longevity relationships for various pollen types. Longevity must be known in order to ascertain the cost and benefits of genebanking pollen.

## Conclusion

There are abundant reports in the literature of many successes for testing pollen viability and temperature exposure. Many of the reported data and methods are difficult to replicate when basic parameters such as initial water content, equilibrated (desiccated) water content and rehydration methods are not described (Dafni and Firmage 2000). It is clear that the physiological state of the pollen at the time of collection and the handling of that pollen within the first few days after collection will determine its potential for long-term survival under optimum conditions. Detailed reporting of handling upon harvest is essential if standardized methods are to be developed. Confirmation that reported staining, *in vitro* germination and *in vivo* germination results are correlated increases confidence in and the repeatability of the reported data.

Despite the challenges, pollen is a valuable genetic resource for conservation. It provides breeders and researchers with an additional, complementary, propagule that may be immediately useful in their programs, although the feasibility of pollen collection and preservation varies among plant species.

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