Collecting pollen for genetic resources conservation

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Introduction

Pollen grains are the mature male (haploid) gametophyte of higher plants. These tiny organs (10–100 μm in diameter) develop in the anthers (Stanley and Linskens, 1974). When the flower opens the anthers become exposed to the environment and dehydration triggers a mechanism for release of the pollen. In the process, the pollen also dehydrates. Though the pollen of most species can withstand drying (Stanley and Linskens, 1974; Towill, 1985; Hoekstra, 1986; Hoekstra *et al.*, 1989b), it may lose some vigour and viability before it lands on a compatible female receptive surface, depending on the mode of dispersal, availability of vectors and environmental factors such as humidity and temperature. There, it produces a tube after rehydration, and this grows into the female tissues to ultimately deliver its two sperm cells to the embryo sac.

Their small size and desiccation tolerance render pollen grains particularly suitable for storage. In fact, pollen storage is a common practice in breeding programmes to bridge the gap between male and female flowering time and to improve fruit setting in orchards (Franklin, 1981; Towill, 1985). However, relatively limited use has been made thus far of pollen for long-term germplasm conservation. One reason for this is the short longevity of pollen grains relative to seeds. Longevity varies widely among species. Also, methods for testing viability are generally cumbersome and time-consuming. Furthermore, there is limited experience in the survival and fertilizing capacity of cryopreserved pollen that is more than five years old (Towill, 1985). Another serious drawback of using pollen in germplasm conservation for the time being is that mature pollen grains cannot develop independently into whole plants.

The advantages and disadvantages of the use of pollen for germplasm storage are discussed further below. The different factors that

influence the rate of loss of pollen viability are then reviewed, followed by techniques for collecting and handling pollen in the field. Sampling strategies for pollen (Namkoong, 1981) are not dealt with in great detail, because they are essentially similar to those for seeds (Chapter 5), except as regards numbers of individuals sampled per site. Finally, methods for viability and vigour assessment are described, and a brief review is provided of storage pollen techniques.

Advantages of the use of pollen

Difficulties can be encountered with the seeds of some species that do not arise with their pollen. Examples of problems associated with seeds are dormancy and special light and cold requirements for germination and recalcitrance (Harrington, 1970; Roberts, 1975). A more general advantage of pollen grains over seeds is their relatively small size. The average diameter of pollen grains is 30–40 μm, which means that very large numbers can be collected and conserved (Akihama and Omura, 1986). Quite apart from the obvious advantage of better genetic representation, access to vast numbers of haploid individuals enables the breeder to make use of pollen selection for polygenic traits from the very first pollination. This is done by applying a surplus of pollen on styles under the conditions to which it is hoped to obtain tolerance, e.g. extreme temperatures or drought (Mulcahy, 1984). Pollen selection is the phenomenon of genetically better adapted tubes growing faster than others through the style, thus preferentially occupying the ovules (Mulcahy, 1979). Pollen selection can occur because a large number of structural genes are expressed in both the sporophytic and the gametophytic phase of the life cycle (reviewed by Mascarenhas, 1989).

The pollen of most species is desiccation tolerant, i.e. it can be dried to a moisture content (MC) of less than 5% on a dry weight (DW) basis, which can be produced by exposure to a relative humidity (RH) of approximately 30% (for reviews, see Towill, 1985; Hoekstra, 1986). This makes it very easy to store the material below 0°C for long periods, without the usual prerequisites for cryogenic storage of fully hydrated plant tissues. However, there are also species that cannot be dried without loss of viability. Species with recalcitrant pollen can be found in the Gramineae (Knowlton, 1922; Goss, 1968), Cucurbitaceae (Gay *et al.*, 1987) and Araceae (Henny, 1980). The expectation is that species with recalcitrant pollen will be particularly common in very humid climates and niches. Species with recalcitrant seeds do not necessarily have recalcitrant pollen, and vice versa.

The international transfer of germplasm in the form of dry pollen is not generally restricted. While literature on the transfer of fungal and bacterial diseases through pollen is scarce, the case of viruses is different (Neergaard, 1977). Nineteen viruses have been shown to occur on the pollen wall or in the cytoplasm and to be able to infect the developing

seed (Mandahar, 1981; Mandahar and Gill, 1984). If the virus survives the development and maturation of the pollen, it may reach the embryo sac via the pollen tube. Although pollen with virus in its cytoplasm seems less vigorous than uninfected pollen, there is still the chance that seeds could become infected. While pollen viruses borne externally have no epidemiological importance, a few of the internally borne viruses have. Therefore, in working with pollen as a source of germplasm, one should be alert to the fact that viruses and other plant diseases might be transmitted during fertilization (Miller and Belcher, 1981).

Disadvantages of the use of pollen

In a considerable number of plant species, the amount of pollen per flower is very small, not really sufficient for effective collecting and processing. Examples are cleistogamous plants and pioneer plants that are self-pollinating (Cruden, 1977). In such cases, pollen storage is unlikely to be a suitable method for germplasm conservation.

Another disadvantage of the use of pollen is a consequence of cytoplasmic inheritance. The number of mitochondria in the sperm cells differs among species, and plastids are usually absent (Wagner *et al.*, 1989; Theunis *et al.*, 1991). The fate of the organelles after fusion of the sperm cells with the egg cell and central cells is unclear. If the organelles survive the fusion events, they are usually outnumbered by the organelles of the egg cell. This would mean that the transmission of organelle genomes is not effective via pollen. This could be a drawback if pollen storage is the only method employed for germplasm conservation in a species. Sex-linked genes will similarly be missed in dioecious species.

In contrast to seed samples, a pollen sample cannot easily be renewed after a time in low temperature storage because pollen cannot independently develop into a plant. Therefore, new collections have to be made. An alternative is for the stored pollen to be used in pollinations with a particular mother, after which the resulting seeds or pollen from flowers of the first cross can be collected and stored, but the original material is then lost. However, it is probably only a matter of time until techniques become available for regrowth of pollen into whole plants. For example, work on the isolation and longevity of sperm cells from the pollen of various species has progressed so far in recent years that it is conceivable that it will soon be possible to force these cells into an embryonic or vegetative type of development (for a review, see Theunis *et al.*, 1991).

The life span of pollen is considerably shorter than that of seed, ranging according to species from just a few hours to several months at room temperature (Pfundt, 1910; Stanley and Linskens, 1974; Shivanna and Johri, 1985). The longevity of the pollen of a number of important crop species is shown in Table 25.1. Pollen of most species is also very

Table 25.1. Longevities of dry pollen (4–10% MC, DW basis) at 20–25°C and normal atmospheric conditions. Longevity is expressed here as the number of days until half or less of the original viability is left. Species are catagorized according to their respective families and to the number of nuclei in pollen.

Family	Species	Longevity (d)	Author(s)
Pinaceae	*Pinus montana*	272	Pfundt, 1910
	Pinus pinaster	275	Pfundt, 1910
	Pinus silvestris	279	Pfundt, 1910
	Pinus strobus	413	Duffield and Snow, 1941
Families with bi- or tricellular pollen			
Araceae	*Arum maculatum* (3)	46	Pfundt, 1910
	Dieffenbachia maculata (3)	2	Henny, 1980
	Spathiphyllum floribundum (2)	14	Henny, 1978
Rubiaceae	*Coffea* spp. (2)	25	Ferwerda, 1937
Families with bicellular pollen			
Aceraceae	*Acer pseudoplatanus*	55	Lichte, 1957
Caricaceae	*Carica papaya*	7	Ganeshan, 1985
Liliaceae	*Lilium bulbiferum*	142	Pfundt, 1910
	Lilium longiflorum	130	Hoekstra, 1986
	Narcissus poeticus	83	Hoekstra, 1986
	Trillium sessile	102	Holman and Brubaker, 1926
Malvaceae	*Abutilon darwini*	14	Pfundt, 1910
	Gossypium hirsutum	2	Rodriguez-Garay and Barrow, 1986
Palmae	*Elaeis guineensis*	107	Hoekstra, 1986
	Phoenix spp.	275	Visser, 1955
Papaveraceae	*Papaver rhoeas*	36	Hoekstra, 1986
Papilionaceae	*Arachis hypogea*	8	Vasil, 1962
	Lupinus latifolius	118	Holman and Brubaker, 1926
	Vicia faba	21	Pfundt, 1910
Rosaceae	*Prunus padus*	181	Pfundt, 1910
	Pyrus communis	114	Visser, 1955
	Pyrus malus	95	Visser, 1955
	Rosa moyesii	57	F.A. Hoekstra, unpublished
Scrophulariaceae	*Antirrhinum majus*	188	Lichte, 1957
	Digitalis purpurea	143	Pfundt, 1910
Solanaceae	*Nicotiana tabacum*	100	F.A. Hoekstra, unpublished

Table 25.1. *continued*

Family	Species	Longevity (d)	Author(s)
	Solanum melongena	50	Vasil, 1962
	Solanum tuberosum	18	Vasil, 1962
Families with tricellular pollen			
Asteraceae	*Chrysanthemum cinerariaefolium*	8	Hoekstra and Bruinsma, 1975a
	Onopordon illyricum	8	Pfundt, 1910
Chenopodiaceae	*Beta vulgaris*	81	Lichte, 1957
	Chenopodium bonus henricus	8	Pfundt, 1910
Cruciferae	*Brassica campestris*	5	Holman and Brubaker, 1926
	Brassica nigra	34	Vasil, 1962
Gramineae	*Alopecurus pratensis*	2	Pfundt, 1910
	Lolium perenne	1	Pfundt, 1910
	Pennisetum typhoides	177	Vasil, 1962
	Secale cereale	0.25	Lichte, 1957
	Triticum aestivum	<1	Vasil, 1962
	Zea mays	1	Pfundt, 1910
Umbelliferae	*Apium graveolens*	6	D'Antonio and Quiros, 1987
	Sanicula bipinnatifida	9	Holman and Brubaker, 1926

sensitive to water (i.e. rain) and cannot be dried after even short exposure to moisture without a considerable decline in viability (Lidforss, 1896; Hoekstra, 1983). There is much disagreement as to the success of different storage protocols (reviewed by Towill, 1985), probably mostly due to the occasionally low vigour of the starting material and to an ignorance of rehydration requirements. This has certainly contributed to the general confusion in the field and reduced the enthusiasm for practical use of cryogenic pollen storage.

Given these advantages and disadvantages, pollen collecting and storage are likely to be most useful in the foreseeable future as a complementary conservation strategy for species that are normally conserved in field gene banks and have large and/or recalcitrant seeds.

Stress on pollen grains

From maturation until the start of tube growth on a compatible style, pollen grains are entirely at the mercy of the environment. Rain,

excessive dryness, extreme temperatures and oxidation all contribute to loss of pollen viability. The focus in this section is mainly on stresses during this independent part of the pollen life cycle. However, climatic conditions can already exert an influence during pollen development in the mother plant. There are reports, for example, that implicate spells of drought, high temperature (Halterlein *et al.*, 1980; Schoper *et al.*, 1987) and low temperature (Maisonneuve, 1983) in the release of bad-quality pollen.

Rain

Continuous rain may prevent anthers from opening. Once pollen has passed physiological maturity, ageing may start within the anthers under such conditions. Ultimately, pollen with reduced viability and vigour is released (Hoekstra and Bruinsma, 1975a; Linskens and Cresti, 1988; Linskens *et al.*, 1989), or the pollen may germinate inside the anthers (Pacini and Franchi, 1982). Rain is also generally detrimental to shed pollen, causing bursting, precocious germination, etc. If pollen is dried after a short period of soaking, it generally loses viability, with a few exceptions among the conifers (Hoekstra, 1983). One might therefore expect plants to protect their pollen from rain, but in a considerable number of species, shed pollen becomes wet during rainy weather (Lindforss, 1896). However, in species where pollen viability is at risk because of an intrinsically short life span or insufficient protection, this is often compensated for by the continuous production of fresh flowers and pollen, for example in a raceme or other type of indeterminate inflorescence (Hoekstra and Bruinsma, 1975a).

Humidity and temperature

Pollen longevity as measured by *in vitro* germination tests has long been known to be strongly affected by RH and temperature in storage (Pfund, 1910; Knowlton, 1922). Desiccation-tolerant pollen of *Typha latifolia* remains viable for about 150 days at 22°C at an internal MC of 6% (on a DW basis), brought about by equilibration in an atmosphere at 40% RH. At 17% MC (equilibrated at 75% RH) this period is reduced to only 16 days. In *Papaver rhoeas*, which has an intrinsically shorter storage life, the survival periods are 45 and four days for the same two MC levels, respectively. Apparently, ageing is considerably faster when MC is high. At 17% MC, respiration in hardly noticeable (Hoekstra and Bruinsma, 1975b), so loss of respiratory substrates is not to blame for this decline in viability. However, lipid breakdown occurs during dry and semidry ageing, most probably associated with the activity of free radicals (McKersie *et al.*, 1988; Bilsen and Hoekstra, 1993). Although the extent of fatty acid unsaturation of membrane lipids hardly changes, some new breakdown products of lipids appear, such as free fatty acids and lysophospholipids. These compounds can cause phase separation in the membranes, which leads to loss of integrity and excessive leakage of endogenous solutes during imbibition (Bilsen and Hoekstra, 1993;

Bilsen *et al.*, 1994). As a general rule, as with seeds (Priestley, 1986), longevity is doubled with every 2% reduction of the endogenous MC (keeping temperature constant).

High temperature reduces longevity as measured in *in vitro* germination tests (Hoekstra and Bruinsma, 1975b). Whereas *Nicotiana alata* pollen at 18% MC (on a DW basis) reaches 50% of its original viability in approximately 400 hours at 20°C, this value is reached in about 80 hours at 30°C. At high humidity, pollen viability in many species is usually at the 50% value after 24 hours at 30°C (Hoekstra and Bruinsma, 1975b). However, before the decline in viability has commenced under conditions of high humidity and high temperature (38°C), a decline in vigour can be observed on account of delayed tube emergence *in vitro* (Shivanna *et al.*, 1991a) and reduced rates of tube growth in the style (Shivanna *et al.*, 1991b). Exposing humid pollen to >45°C will denature the proteins and kill the cells. However, dry pollen is surprisingly tolerant to exposure to temperatures as high as 80°C for 1 hour (Marcucci *et al.*, 1982). Cryogenic temperatures are relatively harmless to dry pollen, whereas they cause injury to humid pollen because free water is turned into ice crystals that pierce the membranes (Towill, 1985). As in seeds (Priestley, 1986), longevity in pollen is doubled for every 5–6°C decrease of temperature.

Desiccation

When pollen is shed from the anthers, it has no control over its MC. A prolonged stay on the flower (e.g. due to the absence of pollinating insects) in combination with dehydrating winds can easily desiccate pollen to <30% MC. Pollen can also dry out on the body hairs of insect pollinators, or during its stay in the air in the case of wind-pollinated species. Already at 30% MC somatic tissue has irreversibly lost its viability. Below 30% MC, membranes and many enzymes undergo conformational changes which lead to loss of integrity and inactivation (Crowe *et al.*, 1987). However, mature pollen of many species is able to withstand an MC of <30%. One of the factors that is responsible for this desiccation tolerance is an elevated disaccharide content (Hoekstra and van Roekel, 1988; Hoekstra *et al.*, 1989a). Experiments have shown that sucrose is effective at preventing or postponing the conformational changes in dehydrating membrane lipids and proteins by replacing the water with -OH groups (Crowe *et al.*, 1987; Hoekstra *et al.*, 1991). Reducing monosaccharides are much less effective in this respect (Crowe *et al.*, 1986). In addition to producing disaccharides, desiccation-tolerant pollen must have mechanisms to withstand the considerable physical forces exerted due to the shrinking of the cell and the extreme solute concentration. It must also effectively cope with the free radicals generated by repressed respiration. These mechanisms are poorly understood at present.

Desiccation tolerance develops at the end of pollen maturation in the anthers (Hoekstra and van Roekel, 1988). Premature collecting may

thus result in material with reduced viability and vigour. Full maturity is assumed to be reached at anther dehiscence in the flower under optimal weather conditions.

One part of desiccation tolerance is obviously the ability of cells to withstand dehydration, but an equally important factor is the successful rehydration of the dry cells. In all desiccation-tolerant organs and organisms, leakage may occur during imbibition of liquid medium, particularly at low temperatures (Hoekstra and van der Wal, 1988). If this leakage exceeds a certain level, reduced vigour results. In particularly severe cases, it is catastrophic for the cell (Hoekstra and van der Wal, 1988; Hoekstra *et al.*, 1992). The suggestion by Simon (1974) that leakage during imbibition of dry seeds is due to transition of the membrane phospholipids from a non-bilayer to a liquid crystalline phase is also believed to hold for dry pollen (Shivanna and Heslop-Harrison, 1981). However, it has been demonstrated recently that leakage coincides with a change from a gel phase to a liquid crystalline phase (Crowe *et al.*, 1989). Methods that frustrate such a phase change during imbibition are effective in reducing the leakage and maintaining vigour. These treatments consist of prehumidification in moisture-saturated air and/or heating during imbibition. Reduction of the rate of water uptake, by using osmotically active substances such as polyethyleneglycol or elevated gelatine and agar concentrations in germination media, is also beneficial.

In seed research there is currently a dispute as to whether storage at ultra-low MC is more or less desirable than storage at a slightly higher level of approximately 5% MC. It has been noticed that in very dry pollen, imbibition at elevated temperatures failed to reduce leakage and restore viability. In fact, leakage became more serious at elevated temperatures of imbibition (Hoekstra *et al.*, 1989b, 1992). This may be explained by the behaviour of the phosphatidylethanolamine. This phospholipid has the tendency to form the above-mentioned non-bilayer, inverted hexagonal (H_{II}) phase at elevated temperatures and reduced hydration levels. Further drying brings the H_{II} transition temperature down, possibly into the physiological temperature region. In the case of very dry pollen, in which phosphatidylethanolamine makes up 30% of the phospholipid content, this may explain why leakage becomes more serious at elevated temperatures of imbibition. While in many species the H_{II} phase may still turn into a liquid crystalline phase by prehumidifying before the germination test *in vitro*, it may be detrimental if the H_{II} phase occurs on too large a scale. All this suggests that storing pollen at far below 5% MC (i.e. in equilibrium with an atmosphere at less than 20% RH) should be avoided.

There are a number of species whose pollen is recalcitrant, i.e. it cannot withstand drying. They can be found in the agriculturally important families Gramineae and Cucurbitaceae and several others. In the case of maize pollen, the endogenous moisture content at which viability is completely lost has been thoroughly investigated. This lies close to 10%

(on a DW basis; Hoekstra *et al.*, 1989a), which is much lower than, for example, a desiccation-sensitive meristem. Below 20% MC there is no freezable water left in the cells, and only tightly bound water is present. This offers possibilities for cryogenic storage in the range 10–20% MC.

Intrinsic sensitivity to ageing

Longevity differs widely among species. Out of 2000 species in 265 plant families, Brewbaker (1959, 1967) found that about 30% shed pollen in the tricellular stage (formerly called trinucleate). These species were mainly in phylogenetically advanced plant families (Brewbaker, 1967; Sporne, 1969). Bicellular (binucleate) pollen grains have still to perform a second mitosis in the style during tube growth, as a result of which the two sperm cells are formed. Families with tricellular pollen are the Asteraceae, Caryophyllaceae, Chenopodiaceae, Cruciferae, Gramineae, Juncaceae and Umbelliferae. It appears that the entire metabolism is relatively advanced in tricellular pollen (Hoekstra and Bruinsma, 1975b; Hoekstra, 1979), which adds to the sensitivity to stresses and ageing conditions. Tricellular pollen tends to be shorter-lived than bicellular, but faster-growing. However, binucleate pollen has been found with short storage life and extremely rapid tube emergence and growth, e.g. in the Balsaminaceae, Cucurbitaceae and Commelinaceae.

Pollen whose lipids are rich in unsaturated linolenic acid (18 : 3) tend to be short-lived and have a more rapid start of tube emergence (Hoekstra, 1986). Pollen linolenic acid content is much higher than in seeds and can reach 80% of total fatty acids (e.g. in the Gramineae and Balsaminaceae). Unsaturated lipids are more prone to peroxidation and breakdown than the more saturated types. The loss of total phospholipids is dependent on both the extent of unsaturation and the amount of lipid-soluble antioxidants present (Hoekstra, 1992). Strangely enough, the short-lived species are particularly low in endogenous lipid-soluble antioxidants. Apparently, longevity of highly unsaturated pollen is not a critical factor in fertilizing efficacy; otherwise this pollen would have been rich in antioxidants. Hoekstra (1992) has suggested that a high level of unsaturation and low level of antioxidants may be prerequisites for rapid tube growth.

The accelerating effect of oxygen on the rate of ageing has been firmly established through longevity experiments at elevated oxygen concentrations (Knowlton, 1922) and after the oxygen had been removed. Jensen (1964) has shown that storage in vacuum considerably extended longevity for all ten of the species he studied, at both 5 °C and room temperature. It is to be expected that pollen with a high proportion of double bonds in its lipids should be particularly sensitive to oxygen. A recent example of dry storage under nitrogen gas at room temperature of such a high linolenic acid type pollen (*Impatiens*) shows that the life span is increased fourfold (Hoekstra, 1992).

Collecting and handling pollen in the field

The main rule to be observed in collecting pollen is to gather material with good viability and vigour. This is simply stated, but, as discussed in the section on stresses, many factors will make it difficult to achieve. Contact of pollen and anthers with rainwater must be avoided, for example. One should thus refrain from collecting on rainy days. Also, only physiologically mature pollen should be collected, which means one should avoid collecting closed anthers from young flower buds. Immature pollen may lack the capacity to germinate, have reduced vigour or still be desiccation-sensitive. Old pollen from previous sheddings should also be avoided. It may be necessary to study the rate of decline of viability of pollen in the particular species and conditions of interest before embarking on collecting. The measurement of viability is discussed in detail in the next section. If, under field conditions, pollen viability has to be estimated without previous knowledge of specific requirements for germination *in vitro* and without sophisticated equipment, the following simple procedure is recommended. A quick wash of the stigma and style with water or ethanol a few hours after application of pollen on the stigma removes those grains that were unable to grow tubes into the stigma. The percentage of grains that are left is indicative of the functional capacity of the pollen sample. Inspection with a simple stereoscopic microscope at low magnification ($\times 40$) will be adequate.

Pollen needs to be dried for conservation. Special care will need to be exercised when collecting species with metabolically active, rapidly germinating, tricellular pollen than with the more quiescent, slowly germinating bicellular types.

Chapter 5 argues that a sample of 59 random unrelated gametes from a population is sufficient to attain the objective of including in the sample at least one copy of 95% of the alleles that occurred there at frequencies greater than 0.05. When collecting seed, this means sampling 30 randomly chosen individuals in a fully outbreeding, randomly mating species, or 59 random individuals in a self-fertilizing species. When collecting pollen, the benchmark criterion will be 59 individuals per population. Self-incompatibility is not likely to cause problems when using collected pollen in crosses. A population of strictly outbreeding, self-incompatible plants must automatically carry a variety of different S-alleles, irrespective of whether the gametophytic or sporophytic system of self-incompatibility is operating (de Nettancourt, 1977). Thus, pollinating a mother plant with the collected material will not lead to mass rejection.

Pollen may be collected directly from plants in the field or from plant parts brought in from outside, say at a base camp with a sheltered table. Outside, pollinators and wind may be serious competitors for the collector, necessitating the bagging of flowers, though temperature and humidity can become very high inside such bags. The collector must also be aware that time of flower opening and anther dehiscence may be

earlier than normal in bags because of the higher temperature (Beers *et al.*, 1981). Pollen should be collected as soon as it is shed. Collecting can be done by shaking flowers over folded sheets of paper or into vials or by using small aspirators.

In some cases, it will be easier to bring pollen-containing plant parts (branches, racemes or just flowers on a stalk) into a sheltered place for collecting. It is advisable not to use cut branches and flowers too long, as pollen quality will decrease as these plant parts age. Supplemental nutrients, sugar and antibiotic compounds may be provided to extend the period during which collecting from cut parts can take place. In some cases, catkins or male inflorescences can be collected and transported for later gathering of the pollen. It is important that this material should not get too hot during transport. Back at base, the material should be spread out and dried by gentle ventilation. This will release the pollen, which can then be separated from the debris by passing through brass or nylon screens before further drying. In a number of species, still-closed anthers can be removed from young flowers or mature flower buds and left to dry. Upon dehiscence, pollen is separated from the anther remainders and cleaned by sieving.

Insects can also be used to collect pollen. Pollen pellets, usually mainly from one species, can be obtained by forcing bees to pass through a screen before they enter the hive. The viability of pollen in the pellets directly after collecting is good, and is maintained after drying and freezing (reviewed by Verhoef and Hoekstra, 1986). However, ageing is accelerated and longevity is reduced by substances added by bees to the pollen, such as reducing sugars, waxes and amylases. An antibiotic occurring in glands in bee heads (10-hydroxy-2-decenoic acid) inhibits pollen germination, but was found not to occur in pollen pellets (Verhoef and Hoekstra, 1986). The main problem with the dried pellets is the difficulty of reusing these hard lumps for pollination purposes. By mixing the pellets with an osmotic solution directly after collecting, filtering the washed pollen and (partly) drying it, a powder can be obtained that is suitable for pollination. However, after these treatments the washed pollen has reduced viability, since it has lost its desiccation tolerance, and will not be suitable for conservation.

It is always important to bring down the MC of the pollen as quickly as possible. Rapid drying can usually be done at 50% RH with gentle ventilation. If ambient RH and temperature are high, as is common in the hot humid tropics, oven drying may be considered (37°C) or the immediate exposure to desiccants such as silica gel or $CaCl_2$ for several hours. The drying should preferably be done at room temperature. Although it would seem appealing to reduce metabolism of the still-humid pollen during drying by exposure to low temperature, there may be a possibility of the rising membrane transition temperature coming close to the dehydration temperature, which may lead to injury. Although no clear evidence exists for this type of injury in pollen, it has recently been found in seeds. Furthermore, during drying at room

temperature, desiccation tolerance may be enhanced due to some synthetic processes that obviously do not occur at low temperature (Hoekstra *et al.*, 1989a).

In general, a moisture content of 5–8% is sufficiently low for the material to be stored in the deep-freeze or at cryogenic temperatures. If pollen is very sticky and not easily removed from the anthers, whole anthers can be dried, which may preserve the viability of the pollen (Crisp and Grout, 1984; Akihama and Omura, 1986). Large pollen samples may require long periods for drying. This may cause a problem, in that pollen that is dried too slowly may start to age, with the result of reduced vigour and viability. In forest tree breeding, special equipment is used for rapid drying and cleaning of the pollen (Sprague and Snyder, 1981). After drying, pollen can be stored in sealed plastic bags containing as little air as possible.

The exception to the rule of rapid drying, of course, is recalcitrant pollen. In grasses, for example, inflorescences can be put in vases and covered with plastic bags. Regular removal of the bags allows collecting of pollen of a homogeneously high MC. Pollen can then be stored, well covered, at low temperatures above 0°C, or first dried in a controlled way for physiological experiments or cryogenic storage (see below).

Methods for viability and vigour assessment

Several tests of pollen viability and vigour exist. Generally, the more accurate the test, the more time it takes to carry it out, with *in situ* pollination and subsequent determination of the quality of the resulting seeds as the ultimate test. The tests described here are mainly laboratory-based. A simple washing technique to remove non-functional pollen, suitable for use in the field, has already been described (see the previous section).

Staining tests

Acetocarmine and cotton blue are among the oldest stains used to assess pollen quality. Positive staining indicates the presence of cytoplasm and nuclei. Whereas lack of staining shows that pollen is non-viable, a positive staining is only a vague indication of viability because there are many factors that leave the cellular content intact but are nevertheless detrimental to survival. If pollen contains high levels of proline, as in maize and rye pollen, the content can be monitored cytochemically by using isatin reagent (Palfi and Köves, 1984). Low viability is associated with reduced proline levels and lacks the intense black staining.

Other stains, such as tetrazolium salts (Hauser and Morrison, 1964; Norton, 1966) and fluoresceindiacetate (FDA) (Heslop-Harrison *et al.*, 1984), are more accurate, results correlating closely with viability as determined by *in vitro* germination tests. However, there is a risk of

false negatives if the following rules are not properly observed. Lipid-phase transitions should be prevented from occurring in the pollen membranes during imbibition in the staining solution (Crowe *et al.*, 1989). This can be achieved by rehydrating the dry pollen in water vapour prior to the test, which considerably increases the percentage of stained grains (Visser *et al.*, 1977; Shivanna and Heslop-Harrison, 1981; Hoekstra and van der Wal, 1988). The duration of the prehydration treatment depends on the species (from a few minutes to several hours). Too long a prehumidification may lead to ageing-associated reduction of membrane integrity, the manifestation of which is leakage of endogenous solutes from the grains and reduction of percentage staining. In such cases, the water vapour treatment should be done at low temperature, for example at 1 °C (Hoekstra, 1979; Akihama and Omura, 1986). Protecting the membranes by prehydration is necessary because small molecules are involved in most staining procedures, which may leak out during the assay. Tetrazolium salts, for example, are acceptors of electrons from active dehydrogenases, during which reaction the water-insoluble coloured formazans are produced. The activity of the dehydrogenases depends, of course, on their molecular integrity, but also on the availability of the endogenous substrate, succinate. Loss of this substrate by improper prehydration can thus give false negatives. Non-viable pollen can give excellent formazan production if succinate is added to the staining solution.

The tetrazolium test differs from older staining methods such as the acetocarmine test in that extra information on the integrity of the pollen plasma membrane is obtained. The same is true for the vital stain, FDA (Heslop-Harrison *et al.*, 1984). This apolar compound readily passes through membranes into the pollen. The non-fluorescent FDA is de-esterified in the cytosol by endogenous esterases, yielding a polar fluorescent dye (fluorescein), which accumulates inside the grain unless the plasma membrane is leaky. Optionally, FDA can be applied in combination with other dyes such as lissamine green or propidium iodide, which cannot normally pass through a membrane unless it is leaky. Thus, staining by the two dyes is mutually exclusive. Lissamine green and propidium iodide can be used to check the effectiveness of the FDA test.

Tetrazolium and FDA have to be administered to the pollen in a suitable germination medium, or at least in a medium with an appropriate osmoticum, in order to protect the pollen from osmotic shock, which could cause membrane leakage. Lack of correlation between extent of staining and *in vitro* germination, which is commonly reported in the older literature, is probably due to membrane injury during application of the stains resulting from improper prehydration. The FDA test can show that membranes are sound but is not proof of germinability. Hydrated immature pollen grains, for example, stain very well, but may not yet have the capacity to form pollen tubes. The good correlation in aged pollen between percentage staining and germination

is due to the dominant role of lipid breakdown in the ageing processes (Bilsen and Hoekstra, 1993; Bilsen *et al.*, 1994).

***Germination* in vitro**

Germination in an *in vitro* system is superior to staining as a viability test. During the 18th century, it was found that pollen could be cultured in relatively simple media to form pollen tubes. Since then, numerous media and culture techniques have been published (for reviews, see Stanley and Linskens, 1974; Shivanna and Johri, 1985). These media usually contain boric acid and salts (Brewbaker and Kwack, 1963) and, depending on the species, different amounts of sucrose and agar or other osmotically active compounds. Media are often adjusted to pH 5–8, again depending on the species. In a number of cases other additives enhance germination and tube growth, e.g. amino acids (Palfi and Köves, 1984; Leduc *et al.*, 1990), casein hydrolysate (Mulcahy and Mulcahy, 1988) and micronutrients and vitamins (Leduc *et al.*, 1990). A popular system is the hanging drop technique, in which pollen in approximately 50 µl of a liquid germination medium is left hanging over a small chamber of vapour-saturated air. The small closed system allows continuous microscopic inspection without the risk of evaporation. Alternatively, pollen may be seeded on the partly dried surface of agar medium in Petri dishes or in shaking or aerated liquid media. The method of viability testing through germination *in vitro* is rapid. In general, pollen tubes can be counted within a few hours.

Plants differ in the amount of stigmatic exudate they produce (Bar-Shalom and Mattsson, 1977; Heslop-Harrison and Shivanna, 1977). Pollen of species with stigmas with profuse exudate produces tubes easily in artificial media with low amounts of sugar and agar, most probably because these conditions are very similar to the situation in the exudate. In these species, pollen is usually shed in the bicellular stage of development. Both dry- and wet-stigma species occur in families with bicellular pollen, however. It is considerably more difficult to germinate the pollen of species with dry stigmas because the water relations are more delicate (Bar-Shalom and Mattsson, 1977; Heslop-Harrison and Shivanna, 1977). Tricellular pollen is almost exclusively found in families with dry stigmas. The reputation of tricellular pollen for being difficult to germinate *in vitro* (Brewbaker, 1959) is probably associated with this. The problem of the poor *in vitro* germination of tricellular pollen is further compounded by its rapid ageing and short storage life.

For *in vitro* germination of tricellular pollen, high sucrose concentrations have been applied, up to 40% (Hoekstra and Bruinsma, 1975a), sometimes in combination with polyethyleneglycol (MW = 4000) (Leduc *et al.*, 1990). Alternatively, water availability can be controlled by high (up to 60%) concentrations of gelatine (Kubo, 1955, 1956), by cellophane sheets (Alexander and Ganeshan, 1989) or simply by restricting the addition of moisture (Bar-Shalom and Mattsson, 1977). Germination has been obtained in this way in pollen from species in the Asteraceae,

Cruciferae, Ericaceae, Gramineae and Chenopodiaceae, although pollen tubes remain short in some cases, additional nutrients then being required.

Occasionally, rapid ejection of tube-like structures in water or germination medium is observed, for example in bicellular cotton pollen (Barrow, 1981) and tricellular *Dipsacus* pollen (F.A. Hoekstra, unpublished results). This is an indication of nothing more than an intact plasma membrane, not necessarily of successful *in vitro* germination.

As in the staining tests, dehydrated pollen has to be treated in humid air before seeding it on to the medium. Failure to do so may have been the reason for the common phenomenon of seemingly non-viable pollen recovering in a later test after storage (Holman and Brubaker, 1926). Visser (1955) and Lichte (1957) have demonstrated in detail the beneficial effects of prehydration in humid air on growth performance of pollen *in vitro*. Apart from reducing the gel-to-liquid crystalline transition temperature of membrane lipids, water vapour may stimulate respiration and ageing. This is particularly risky for tricellular pollen.

The ability to form pollen tubes *in vitro* does not give an absolute answer as to whether pollen is vigorous enough for tube growth in the style towards the ovules. There are a number of reports on pollen with good germination *in vitro* failing to fertilize successfully (Shivanna *et al.*, 1991b). Also, pollen from the Umbelliferae, from some species of the tricellular families mentioned above and from a few other families cannot be successfully germinated *in vitro* at all. In such cases, germination *in vivo* is the only method suitable for viability assessment.

***Germination* in vivo**

The most reliable method of pollen vigour determination is the regular monitoring of the progress of the pollen tubes in the style. This can be done by microscopic analysis of styles that are stained with aniline blue or Wasser blue (Linskens and Esser, 1957; Shivanna *et al.*, 1991b). In fleshy styles an additional bleaching in KOH, followed by extensive washing and squashing, has to be done before the fluorescing pollen tubes can be detected. If a fluorescence microscope is not available, it is possible to stain the tubes with an aqueous solution of cotton blue in combination with extensive bleaching (Janson, 1993). Although it remains difficult to determine accurately the percentage of pollen grains showing tube growth, a rough estimate is possible. A promising method is a semi-*in vivo* technique, in which, after pollination, the style is cut and the cut end placed in germination medium (Shivanna *et al.*, 1991b). The number of penetrating pollen tubes and their speed of growth can then be determined.

It is still an unanswered question whether hydration is required prior to pollination with dry stored pollen. Usually, conditions on the stigma will allow gradual rehydration of the pollen. A few papers report that prehydration was not effective, but emphasize that it was not harmful either (Matthews and Bramlett, 1983; Hecker *et al.*, 1986). However,

beneficial effects have also been reported. It can be anticipated that in profuse exudate dry pollen may suffer imbibition injury, which would have been prevented by prehydration in humid air. Prehydration may also help in rendering pollen more adhesive, facilitating attachment to the receptive surfaces.

In vivo tests can be done in a few days. If time is not a critical factor, the maturation of seeds can be awaited, and their germination tested, followed by screening of seedlings for normal growth performance.

Pollen storage methods

Pollen storage methods will not be discussed in great detail here, as some excellent reviews are already available (Binder *et al.*, 1974; Stanley and Linskens, 1974; Franklin, 1981; Shivanna and Johri, 1985; Towill, 1985). For practical aspects, the reader is referred to the appropriate chapter in the *Pollen Management Handbook* (Matthews and Kraus, 1981; Schoenike and Bey, 1981). For information specifically on woody perennials, see FAO (1993). What emerges from these studies is that desiccation-tolerant pollen generally does not survive longer than about one year at room temperature in the desiccated condition under normal atmospheric oxygen pressure. For longer storage the sample must be cooled or the oxygen removed. Storage of dry samples at −20°C or lower can only be done successfully at below 20% MC (on a DW basis). A higher initial MC will lead to the deleterious formation of ice crystals. Storage in organic solvents is a promising option (Iwanami and Nakamura, 1972; Iwanami, 1973; Yabuya, 1983; Jain and Shivanna, 1990). For long-term storage (i.e. several decades or more) cryogenic storage is required. Storage longevity will be essentially infinite in liquid nitrogen (Bredemann *et al.*, 1947). For such storage, pollen must always be desiccated in advance, perhaps by freeze- or vacuum-drying (Towill, 1985).

The pollen of grasses is notorious for its poor storability. This is due to its sensitivity to dehydration, the genus *Pennisetum* being an exception. In practice, this means that such pollen is stored at high MC which limits survival to days rather than weeks due to high metabolic activity and the associated ageing (extensively reviewed by Shivanna and Johri, 1985). A slight extension of longevity can be achieved by storage at low, above-freezing temperature. Some grass pollen can nevertheless be dehydrated to some extent. Maize pollen, for example, can be dehydrated to such a low MC that freezable water is removed (Kerhoas *et al.*, 1987), while still remaining viable as determined by *in vivo* germination (Hoekstra *et al.*, 1989a). Exploiting this, Barnabas and Rajki (1976, 1981) partially dried maize and rye pollen and stored it in liquid nitrogen and at −76°C. At the time of cooling, the pollen was at approximately 20% MC (on a DW basis). After rapid thawing it gave tube growth on the silks and seed set. This was true for both temperature conditions of

storage at which the pollen was kept for at least three years. It is essential that, after application on the silks, the pollen is not further dried out. In other recalcitrant pollen the ability to survive partial drying may equally offer the possibility for long-term storage. If partial drying is not tolerated, the usual cryogenic protocols with controlled slow cooling and the use of cryoprotectant solutions might be carried out. However, there is hardly any experience with these methods in pollen (Towill, 1985).

Examples of pollen collecting

Most examples of pollen collecting have taken place as part of a programme aimed at the introduction of new genes into established crop species. Three case-studies are examined below, with the delay between collecting and pollination of mother plants ranging from a few days to three years.

***Pyrethrum* (Chrysanthemum cinerariifolium, *Asteraceae*)**

Pyrethrum is grown for its insecticidal pyrethrins, which are extracted from the dried flower heads. It was introduced into Kenya in 1928 from semiwild plants growing along the Dalmatian coast. To broaden the supposedly narrow genetic base of the crop, pollen and seeds were collected in 1971 from 18 sites along the entire coast (Parlevliet *et al.*, 1979). Pollen longevity under dry conditions at room temperature was investigated by *in vitro* testing prior to collecting (Hoekstra and Bruinsma, 1975a) and found satisfactory for bridging the few weeks between collecting and pollination.

The collector faced intense competition from pollinating flies at the collecting site. Just as pollen began to be shed in the morning, it was tipped from the flower-heads into small vials and immediately dried for 12 to 14 hours over NaOH pellets at ambient temperature. The pollen samples were then packed in small plastic bags and sealed with as little air as possible. The bags were sent to Kenya by airmail. The viability of the dried tricellular pollen was still such that successful pollinations could be made with local clones of good general combining ability. The pollinations were made by hand (using a brush) in a greenhouse at high temperature and high RH.

Pyrethrum is self-incompatible, so that the profuse seeds derived from these pollinations had certainly received the Dalmatian genes via the foreign pollen. Plants grown from introduced seeds gave serious problems because they remained vegetative under Kenyan climatic conditions. In this respect, pollen collecting proved superior, since the plants derived from pollination with the introduced pollen gave few such problems.

Oil-palm (Elaeis guineensis)

The collecting and storage of oil-palm pollen was carried out in the context of breeding programmes (Ekaratne and Senathirajah, 1983). Male inflorescences with flowers in which the anthers had just begun to dehisce were shaken to release pollen on to paper sheets. The pollen was cleaned from adhering debris by sieving it through a 0.5 mm screen. The MC content was above the critical value of 20% (on a DW basis), so additional drying was required before freezing. This was done by placing the pollen for 2–8 hours in an oven at 37°C and then transferring it to airtight bottles at −15°C.

After four months of storage, viability as measured by *in vitro* germination was still approximately 90%. The simple germination medium consisted of sucrose (10%), agar (0.5%) and 100–200 ppm boric acid. The pollen was applied to a drop of the medium kept at high RH. Receptive stigmas were pollinated with pollen stored for 12 months at −15°C. Pollen survives for about six days on the flower. The female inflorescences were bagged before and after pollination to prevent contamination. Total fruit weight did not differ from control pollinations with fresh pollen, from which it can be concluded that oil-palm pollen can be stored dry for at least one year in an ordinary deep freezer and retain its fertilizing capacity.

Pecan (Carya illinoensis)

In pecan, pollen was bottled for cryogenic storage (Yates and Sparks, 1989, 1990). Catkins with dehiscing anthers were brought into the laboratory and spread on paper. Pollen was collected after 24 hours, during which time sufficient dehydration had taken place. Debris was removed by filtering the pollen through a brass screen. Pollen was then placed in cryovials and stored at −80°C and −196°C in liquid nitrogen.

Pollen survived at both temperatures for three years, as confirmed by *in vitro* germination and *in vivo* pollination and subsequent fruit set. Prehydration in humid air for 4 hours is required for successful *in vitro* tests. Germination *in vitro* was performed in a simple medium containing 15% sucrose, 150 ppm boric acid and 500 ppm $Ca(NO_3)_2 \cdot 4H_2O$. In the pollination experiments, the female flowers were carefully bagged to prevent contamination. The equally high fruit set after pollination with stored and fresh pollen indicates that cryogenic storage can be used for long-term germplasm conservation in pecan.

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