Pollen viability in the field

M. Bots and C. Mariani

Radboud Universiteit Nijmegen

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Summary

Pollen represents a critical stage in the life cycle of plants, as viable pollen is crucial for efficient sexual plant reproduction. However, pollen may also be a vector for transgene escape from genetically modified crops. To investigate the possible contribution pollen viability may play in determining transgene escape, the project described in this report had two objectives: to review the available literature on pollen viability and the effect of environmental factors on it, and to analyze the viability of pollen of various species in the environment.

Reviewing scientific literature on pollen viability it was found that pollen viability is mainly affected by drought/dehydration, heat stress and UV-B radiation. These factors not only affect pollen viability after dehiscence, when the pollen is exposed to the environment, but also during its development inside the anther. The effect of each factors affects pollen viability in a species-specific way, depending on the physiology of the pollen grains and the presence or absence of specific structural modifications. A complicating factor for the comparison of various experiments was the absence of standardized protocols and experimental conditions used. Assessing the contribution of pollen viability to transgene escape is further complicated by the fact that much of the literature described experiments that were performed in greenhouses with greenhouse-grown plants, and investigated the effect of a single factor only. Although informative, these experiments do not represent field situations and cannot be extrapolated to field situations without further experimental support. In conclusion, although considerable data is present is available on pollen viability, it is difficult to use it for the prediction of the contribution of pollen viability to transgene escape.

The second goal of the project was to analyze pollen viability in the field. Three species were chosen for analysis: Brassica, strawberry and wheat. For all species, pollen viability was analyzed using *in vitro* pollen germination, as this method is widely used and accepted and can be quickly performed in the field.

For **Brassica**, it was found that pollen retains some viability for at least 72 hours. As for this species pollen is mainly transported by bees that visit young flowers more regularly than older flowers, it is expected that generally fresh pollen is collected.

For **strawberry** it was found that pollen viability drops faster than for Brassica, although the pollen of these species share physiological characteristics. After 6 hours, strawberry pollen had lost approximately 50% of its viability.

For **wheat** no conclusions could be drawn. This was caused by problems with the collection of freshly dehisced pollen. In addition, *in vitro* germination is less efficient for this species further complicating interpretation of the results.

With respect to the contribution of pollen viability to determining transgene escape efficiencies, it is concluded that current knowledge is insufficient. However, we believe that knowledge of pollen viability is not sufficient to predict transgene escape, but that out crossing rates should be directly measured. This can for example be done by using male sterile bait plants and sensitive techniques to detect out crossing events, as was shown by various large scale experiments referred to in the report.

Part 1 Literature Review

1 Introduction

In recent years increasing numbers of transgenic crops have been introduced into the environment worldwide, although not as much in The Netherlands. The use of such crops can be advantageous for various reasons, but has also raised concerns with respect to the possible escape of transgenes into related wild species or other crops. As many of the transgenic lines used in the field contain herbicide resistance genes, such events may result in the formation of weeds that are herbicide resistant. Alternatively, the presence of transgenes in wild species may harm non-target species, may have disruptive effects on biotic communities or may lead to genetic erosion (Ellstrand, 2001).

Transgene escape can take place through vegetative reproduction, or using seed or pollen as vector. Seed spillage may cause the formation of stable feral populations, especially when the environmental conditions are advantageous and the level of competition is low. Pollination may result in crosses between transgenic and non-transgenic lines of a given crop or alternatively in the formation of hybrids between related wild species. If such hybrids are fertile and backcrosses occur, this may result in introgression of foreign genes in the recipient population.

The formation of hybrids between related species is a common phenomenon (Ellstrand et al., 1999). In the United Kingdom, more than 700 different hybrids have been observed on a total of 2500 natural species (Raybould and Gray, 1993). Furthermore, in a case study on hybrid formation in the world's 13 most important food crops, substantial evidence was found for hybridization between ten of the crops and wild relatives was (Ellstrand et al., 1999). Recently, calculations performed on the basis of population surveys, pollen dispersal profiles, herbarium data and other sources, indicated that on a yearly basis approximately 49000 hybrids may be formed between *Brasscica napus* and *B. rapa* in the United Kingdom (Wilkinson et al., 2003). The examples mentioned above indicate that the formation of hybrids is likely to be more a rule than an exception. In fact, it has been suggested that hybrid formation may be an important factor in species evolution (Ellstrand et al., 1999; Arnold, 2004).

The rates with which hybrid formation occurs are only known for a limited number of species and geographical areas, and appear to vary considerably (de Vries et al., 1992). Factors influencing hybrid formation rates include the vicinity of related (wild) species, pollen viability and the presence of suitable pollination vectors, efficiency of seed set and seed viability and finally hybrid fitness and fecundity.

Recently, considerable attention is given to a number of these factors, but the influence of pollen viability has received relatively little attention (de Vries et al., 1992; Eijlander and Stiekema, 1994; Groot et al., 2003; Ramsay, 2003). However, given the importance of pollen development and function in sexual plant reproduction, it is obvious that pollen viability is a prerequisite for hybrid production. Therefore, knowledge of pollen viability and the way it is affected by environmental factors may help predicting hybrid formation. Here, we present a literature review of factors that affect the viability of pollen during its development in the anther and when exposed in the environment.

2 Pollen development and functioning

Pollen grains are produced in specialized floral organs called stamens, which consist of a stalk with vascular tissue and a two-lobbed anther. They develop inside the anther until it splits and the mature grains are released into the environment (dehiscence). Pollen grains then have to be transported to a compatible stigma and they do so using one of various possible vectors. Common vectors for pollen transport are wind and various insects, depending on the species. When a stigma is reached, the pollen grain forms a pollen tube that will grow through the tissues of the pistil towards the ovary to deliver the sperm cells.

Pollen development inside the anther is complex and involves various processes that occur simultaneously to enable pollen development on one hand, and that prepare the anther for pollen release on the other. One special feature of pollen grains is their wall, which is completely different from the wall of any other plant cell. The pollen wall is multilayered, derived from both the developing pollen and sporophytic cells of the anther and consists of material that is highly resistant to degradation, making it an important determinant of pollen viability. In some species, on the surface of the pollen wall a layer of material is present, known as the pollen coat, tryphine or pollen kit (Taylor and Hepler, 1997). This sticky substance may contain lipids, proteins and phenolic compounds, and is especially substantial in entomophilous species. The functions of the pollen coat are thought to include sticking to insects to facilitate pollen transfer and protection against UV-radiation, but the lipids and proteins of the pollen coat also play an important role on the stigma (Dickinson et al., 2000).

In many species, pollen grains undergo programmed dehydration before release from the anther. Dehydration of the pollen is accompanied by dehydration of the anther cells and by sugar-starch conversions in both (Keijzer, 1987; Pressman et al., 2002). After dehiscence, the pollen of many species is dehydrated and metabolically inactive. In some species, however, pollen grains are still partially hydrated and metabolically active, which enables fast pollen tube formation (Heslop-Harrison, 2000; Nepi et al., 2001). When a dehydrated pollen grain arrives on a compatible stigma, it takes up water from it and produces a pollen tube. Depending on the species, this process may take from minutes to several hours. Once formed, the tube enters the pistil and is shielded from the environment, and dependent on resources provided by the pistil to accomplish fertilization. However, both on the stigma surface and in the pistil molecular mechanisms

may be functional that prevent fertilization by genetically distant or closely related pollen (McCubbin and Kao, 2000).

3 Assessing pollen viability

Considering the function of the pollen grain in the life cycle of a plant, one way to test pollen viability would be to use the pollen for pollination and subsequently analyze seed set. However, because this is time consuming and often not feasible, other methods are frequently used to assess pollen viability. Table 1 lists some of the most commonly used methods, a more extensive overview has recently been provided by Dafni and Firmage (2000).

Table 1. Methods to determine pollen viability

Method	Advantage	Disadvantage
Various methods (X-Gal, Baker's reagent, benzidine test, peroxidase indicator)	Variable, dependent on test	Variable, usually applied for few species only
Vital stains (FCR, TTC)	Very fast, applicable to virtually all species. Tests cytoplasmic enzymes and in some cases integrity of plasma membrane.	False positives reported repeatedly, not always correlation with seed set
In vitro pollen germination	More accurate, easy protocol for most species	Not all species germinate easily, optimum germination medium may differ between species. In vitro germination rates may be lower than on stigma. Not always good correlation with seed set, but generally more reliable than vital stains.
In vivo pollen germination	Simulates natural pollination, more valid than in vitro germination	Laborious. Compatibility mechanisms may prevent germination in certain species
Seed set	Most natural and reliable method to examine pollen viability	Laborious. Hand-pollination may lead to over-estimation of pollen viability

The fastest way of analyzing pollen viability is using vital stains that react with pollen enzymes, thereby indicating the presence of intact cellular contents. The most commonly used vital stain is the fluorochromatic reaction (FCR) which reveals esterase activity in pollen with an intact plasma membrane (Shivanna and Heslop-Harrison, 1981). This test can be performed very quickly in the laboratory, and often the pollen viability results correlate with seed set. However, in a number of experiments false

positives were reported or no relation with seed set could be established. In general, vital stains frequently produce false positives leading to an overestimation of pollen viability.

Another frequently used method to assess pollen viability is *in vitro* germination. Because pollen grains of many species will easily germinate in a medium that contains boric acid and an osmoticum, this method is widely used (Taylor and Hepler, 1997). Furthermore, in recent years protocols have been developed that allow high germination frequencies of pollen of the more recalcitrant pollen. However, despite the simple basic requirements of pollen tube growth media, the optimal composition may vary from species to species and the use of suboptimal media may underestimate pollen viability. Still, pollen germination rates usually provide more reliable data on pollen viability than vital stains.

Finally, pollen viability may be measured after pollination, by analyzing germination on the stigma or seed set derived from that pollination. Both methods are time consuming and may lead to an overestimation of pollen viability if the pistil is over-pollinated.

Although all methods are valuable with respect to predicting seed set, they are prone to errors and results should be interpreted carefully. Furthermore, obtaining accurate pollen viability rates may depend strongly on storage conditions, protocols used and other factors. Because of these reasons, it is difficult to compare the results obtained in individual experiments.

3.1 Factors influencing pollen viability

Pollen viability may be affected at different stages of development, from early in the anther till late on the stigma. The most direct interaction between pollen grains and the environment occurs after release from the anther. As expected, most of the factors that influence pollen viability do so at this stage. However, in some cases the occurrence of various stresses during pollen development inside the anther may strongly affect pollen viability. Pollen tube growth in the pistil, by contrast, appears to be relatively insensitive to stresses.

The literature on pollen viability that appeared until 1974 has been reviewed by Stanley and Linskens (1974). The accumulated data indicated that pollen viability was influenced by relative humidity, temperature, atmospheric composition and oxygen

pressure. General trends, listed in table 2, included the observation that pollen of most species remained viable longer when stored at low relative humidity, a notable exception being pollen of Gramineae.

Table 2. Factors affecting pollen viability during storage (Stanly and Linskens, 1974)

Factor	Effect
Relative humidity	Low humidity during pollen storage usually has a positive effect on pollen viability. Notable exceptions include species of the Gramineae family
Temperature	Storage at low temperatures usually has a positive effect on pollen viability
Atmosphere composition	Increased CO has a positive effect of pollen viability after storage
Oxygen pressure	Decreased oxygen during storage has a positive effect on pollen viability

Furthermore, at low temperatures pollen remains longer viable in most species analyzed (Stanley and Linskens, 1974). However, most experiments were designed to study the storage of pollen and showed little data on pollen viability in the field.

Only recently the research on pollen viability has focused more on negative effects of some factors that may be present in natural environments. It is not surprising, however, that the same factors that affect pollen storage also have an effect on pollen viability in the field. Table III lists the most recent data on factors affecting pollen viability, grouped per species.

3.1.1 Humidity

The effect of relative humidity of the environment on pollen viability is apparent in many species investigated. The response to high or low humidity, however, may differ between species and is usually associated with the intrinsic hydration state of the pollen at dehiscence (Nepi et al., 2001). Pollen of most species contain very little water (Stanley and Linskens, 1974), but some pollen grains have relatively high hydration levels. A well known example is the pollen of the Gramineae, which together with more than 40 angiosperm families contain more than 30% water at dehiscence (Franchi et al., 2002). Besides having higher hydration levels, these pollen grains are also metabolically more active, which allows fast pollen tube extrusion (Heslop-Harrison, 2000). Usually, pollen grains with higher water content are more sensitive to low relative humidity (RH) in the environment because they lose water more rapidly. In such desiccation intolerant species loss of water is thought to lead to irreversible changes in the pollen membranes

(Shivanna and Heslop-Harrison, 1981; van Bilsen et al., 1994). To minimize water loss to the environment, pollen grains of various species have developed adaptations. These may involve structural adaptation of the pollen wall or the presence of sucrose in the pollen cytoplasm to reduce water loss and protect membranes (Hoekstra et al., 1991; Speranza et al., 1997; Heslop-Harrison, 2000).

Experimental analysis of the consequences of dehydration for pollen viability has been performed most thoroughly for maize pollen. When exposed to dehydrating conditions of the atmosphere, maize pollen grains lose virtually all viability within approximately 3 hrs, as measured by *in vitro* germination of the treated pollen grains. The exact time before all viability was lost depended on the humidity during the experiment: at 20% RH, for example, all viability was lost within 50 minutes, whereas at 75% RH some pollen were still viable after 4 hrs (Luna et al., 2001; Aylor, 2004). Exposure to the environment was associated with a progressive loss of water from the pollen, and germination was severely inhibited at water content values of 30% and lower (Aylor, 2003). During the experiment, the maize pollen grains turned from spherical and white to collapsed and yellow, reflecting the progression of the dehydration process (Luna et al., 2001; Aylor, 2003).

An analysis of pollen viability using FCR in eight different species after short term storage at various RH levels was performed by Shivanna and Heslop-Harrison (1981). The results of the analysis indicated that pollen grains of different species responded differently to 4-24 hours of storage. For example, *Secale cereale* pollen stored at low RH lost all viability within 2 hours, whereas at high RH viability was only lost after 24 hours; *Cytisus battandieri* pollen that were stored for 24 hours storage at low RH, by contrast, still displayed viability rates comparable to fresh pollen. In some species, prehydration of the stored pollen increased germination percentages (Shivanna and Heslop-Harrison, 1981). Although it was not directly established that water loss contributed to the reduction of viability in stored pollen, the observation that the decrease in viability was correlated to the relative humidity does suggest that this could be an important factor.

Nepi et al analyzed seven species with partially hydrated pollen (i.e. water percentage >30% at dehiscence), and found that viability after exposure to their environment varied between 10 to 70% (table 3) (Nepi et al., 2001). Some species, like *Cucurbita pepo*, lost all viability within 36 hrs after flower opening, whereas others, like *Opuntia dillenii*, still showed considerable viability after 72 hrs. However, these percentages are based on FCR and actual germination frequencies were reported to be lower (Nepi et al., 2001). Similar variability was found when analyzing pollen viability

after dehiscence of three entomophilous and three anemophilous species (Pacini et al., 1997). After 72 hrs of exposure to the environment, pollen of some species was still viable, whereas others had lost all viability, but this difference was not correlated to the type of pollen dispersal.

It is unclear what causes the differences between species in viability loss during exposure to dehydrating environments, although for some species it has been suggested to be related to the type of carbohydrates that are stored in the cytoplasm (Pacini, 1996). For example, maize only contains 5% sucrose and loses viability rapidly through dehydration, whereas *Pennisetum typhoides* contain 14% sucrose and survives much longer (Hoekstra et al., 1989).

In most species, it is the absence of water that causes loss of pollen viability, but in some cases also the presence of water in the form of rain can be detrimental for pollen. For cotton, for example, it has been shown that water causes pollen to burst. This phenomenon not only has strong effects on boll set, but also provides opportunities to induce male sterility by applying water to flowering plants (Burke, 2002).

Water balance has a strong influence on the viability of mature pollen grains when they are exposed to the environment, but also during the development of pollen inside the anther. Especially in cereals, short periods of drought stress specifically affect male reproductive performance and can greatly reduce grain yield (Saini, 1997). In both wheat and rice, the effect of drought stress is strongest if it occurs during meiosis, but later stages are sensitive as well. The effects of the stress in the anther include changes in sugar and starch concentrations and activities of enzymes of starch synthesis. However, it is not desiccation of the anther that leads to reduction in pollen viability, as it was shown that the water potential of the spikelet was unaffected by the stress (Dorion et al., 1996; Sheoran and Saini, 1996). Morphological analysis of stressed anthers revealed several changes, including the presence of starch in the connective tissue, where it normally is not present. Despite the abnormalities, more than 70% of the stressed anthers contained microspores, indicating that meiosis does take place (Lalonde et al., 1997).

In conclusion, pollen grains of many species may be sensitive to excessive dehydration caused by low relative humidity, but adaptations to prevent such dehydration are species specific. In addition, drought stress during pollen development can also strongly affect pollen viability.

3.1.2 Temperature

Like water status, temperature can affect pollen grains during transport and germination on the stigma, but also during development in the anther. From several studies it appears that mature pollen grains are generally rather resistant to temperature stress applied after dehiscence, but detailed investigations have been carried out in a few instances only. In tomato, applying mild heat stress after dehiscence did reduce fruit set, but the differences with controls were not significant (Sato et al., 2002). More severe heat stress treatments of Brassica pollen revealed that they still germinated after exposure to 60°C for 4 hrs; if the pollen was prehydrated in humid air prior to germination they even germinated after 24 hrs at 45°C (Rao et al., 1992). However, germination rates and pollen tube lengths were significantly lower than for controls. Pollen grains treated at 75°C did not germinate at all, with or without prehydration. Seed set after pollination with pollen treated at 75°C or at 60°C for 24 hrs was reduced, but pollination with any of the other samples led to normal seed set (Rao et al., 1992). One possible explanation for this observation may be that pollen germination on the stigma can overcome effects of the heat treatment, but it is also possible that the stigmas were over-pollinated, thereby masking the reduced germination rates. Furthermore, because the pollinations were performed with treated pollen only, there was no selection on pollen tube growth rate. It is therefore possible that pollen performance may be affected in mixed pollinations as they occur in natural environments. Potato pollen form an exception to the observation that pollen grains are generally rather resistant to temperature stress (Pallais et al., 1988). Exposure of pollen of different potato varieties to 30°C for up to 30 minutes strongly decreased pollen germination, while seed set was less affected. The decrease varied between 30 to 70% for different potato varieties (Pallais et al., 1988).

Temperature stress in the period preceding dehiscence resulted in more severe consequences for pollen viability than after dehiscence. Furthermore, both cold and heat during pollen development can negatively affect pollen viability, depending on the species. In the case of mango trees, for example, cold periods are associated with reductions in fruit set and it was demonstrated that night temperatures below 10°C decreased pollen viability to 50% of controls (Issarakraisila and Considine, 1994).

The effect of heat stress during reproductive development has most thoroughly been investigated in tomato. Temperatures between 20-25°C were reported to be optimal for tomato fruit set (Sato et al., 2002). Surprisingly, raising the temperature to 29°C drastically reduced the number of fruits formed and seeds set. Using male sterile

plants it was shown that this effect was largely caused by defects in male reproductive development, while female development was less affected (Peet et al., 1998). Comparison of five different tomato cultivars revealed that they displayed similar responses to heat stress, although the severity of the effect differed between them. For all cultivars, heat stress resulted in reduced fruit set, a reduction in the number of pollen grains released and a 75% decrease in germination rate of pollen grains (Sato et al., 2000).

Table 3: Factors affecting pollen viability

Species	Treatment/Stress	Analysis	Effect	Reference
Acanthus mollis	Exposure of mature dehisced pollen to air	FCR	Viability decreases constantly to less than 20% after 72 hours	Pacini et al 1997
Allium ostrowskianum	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Alcea rosea	Exposure of mature dehisced pollen to air	FCR	Viability decreases slowly, still 40-60% after 72 hrs	Nepi et al 2001
Althaea officinales	Exposure of mature dehisced pollen to air	FCR	Viability decreases slowly, still 40-60% after 72 hrs	Nepi et al 2001
Arabidopsis thaliana	Heat stress during pollen development	Seed set	Heat stress strongly decreases pollen viability	Kim et al 2001
Arachis hypogaea	Heat stress during pollen development	TTC	Short periods of heat stress decrease pollen viability	Vara Prasad et al 1999
Brassica juncea	Exposure of mature dehisced pollen to heat stress	FRC, germination, seed set	Severe heat stress decreases pollen viability	Rao et al 1991
Brassica nigra	Pollen development and pollination under enhanced UV-B light	Seed set	No reduction in seed set	Conner and Zangori 1997
Brassica rapa	Pollen development and pollination under enhanced UV-B light	Seed set	No reduction in seed set	Conner and Zangori 1997

Table 3, continued

Species	Treatment/Stress	Analysis	Effect	Reference
Capsicum annuum	Development under high temperature; with and without high CO2	Pollen germination	High temperature inhibits pollen fertility, high CO2 reverts effect. Sugars and sugar metabolizing enzymes in anther and pollen affected	Aloni et al 2001
Caryopteris clandonensis	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Chamaerops humilis	Exposure of mature dehisced pollen to air	FCR	Viability remains high for at least 72 hours	Pacini et al 1997
Cleome spinosa	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Cucurbita pepo	Exposure of mature dehisced pollen to air	FCR	Pollen viability decreases slowly during first six hours, than more rapidly	Nepi and Pacini 1993
		FCR	All pollen were inviable after 48 hours	Pacini et al 1997
Cytisus battandieri	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	Viability unchanged after 24 hrs irrespective of RH	Shivanna and Heslop-Harrison, 1981
Digitalis purpurea	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	50% viable after 20 hours at low RH, viability higher at high RH	Shivanna and Heslop-Harrison, 1981
Epilobium	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Erythronium grandiflorum	Exposure of mature dehisced pollen to air	FCR, germination, seed set	Viability declines in time, but results for different analyses are not identical	Thomson et al 1994
Eschscholzia californica	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Festuca arundinacea	Exposure of mature dehisced pollen to air	FCR	Viability remains high for 48 hours, then decreases raidly	Pacini et al 1997

Table 3, continued

Species	Treatment/Stress	Analysis	Effect	Reference
Gossypium hirsutum	Exposure of mature, dehisced pollen to rain/water	Seed set	Water is detrimental for pollen functioning	Burke 2002
Iris pseudacorus	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	No viable pollen after 2 hours at low RH; pollen viability unchanged after 4 hours at high RH	Shivanna and Heslop-Harrison, 1981
Isatis tinctoria	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Lavatera arborea	Exposure of mature dehisced pollen to air	FCR	Viability decreases slowly, still 40-60% after 72 hrs	Nepi et al 2001
Leymus chinensis	Exposure of mature dehisced pollen to air	TTC	Viability drops from 75% (fresh) to 5% after three ours exposure	Huang et al 2004
Lilium	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Lonicera periclymenum	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	Pollen viability decreases fast at low RH; at high RH 50% viability after 24 hours	Shivanna and Heslop-Harrison, 1981
	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Lycopersicon esculentum	Heat stress during pollen development	Seed set	A small increase in temperature abolishes all pollen viability	Peet et al 1998
	Continous mild heat stress	Pollen germination	Pollen germination is reduced by continuous mild heat stress. Reduction variable for different varieties	Sato et al 2000
	Heat stress during pollen development		Decrease in pollen viability is associated with alterations in carbohydrate metabolism	Pressman et al 2002
	Exposure of mature dehisced pollen to mild heat stress	Fruit set	No effect on fruit set	Sato et al 2002
Malus	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998

Table 3, continued

Species	Treatment/Stress	Analysis	Effect	Reference
Mangifera indica	Effect of cold stress during pollen development	FCR	Cold stress reduces pollen viability with time. Meiosis stage most sensitive to cold stress	Issarakraisila and Considine 1994
Mercurialis annua	Exposure of mature dehisced pollen to air	FCR	Viability decreases steadily, all viability lost after 72 hours	Pacini et al 1997
Mirabilis jalapa	Exposure of mature dehisced pollen to air	FCR	Viability decreases slowly, still 40-60% after 72 hrs	Nepi et al 2001
Nemophila maculata	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination; tube length increased	Torabinejad et al 1998
Nicotiana tabacum	Exposure of mature dehisced pollen to heat stress and high humidity	Pollen germination	Heat stress or high humidity alone did not affect pollen viability; combination of the stresses did	Shivanna et al 1991
	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Oenothera caespitosa	Pollen germination under UV-B radiation	Pollen ger-mination, pollen tube length	Pollen germination and tube length significantly reduced	Torabinejad et al 1998
	Exposure of mature dehisced pollen to air	FCR	Viability drops quickly, all pollen dead after 72 hrs	Nepi et al 2001
O. pallida	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen germination and tube length significantly reduced	Torabinejad et al 1998
Opuntia dillenii	Exposure of mature dehisced pollen to air	FCR	Viability unchanged after 72 hrs	Nepi et al 2001
Oryza sativa	Drought stress during pollen develoment	Grain set	Grain set drops to 20%; anther and pollen display alterations in sugar metabolism	Sheoran and Saini, 1996
Phacelia tanacetifolia	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Phaseolus vulgaris	Heat stress during pollen development	Acetocarmine stain	Heat stress affects pollen viability, effect dependent on genotype	Porch and Jahn, 2001

Table 3, continued

Species	Treatment/Stress	Analysis	Effect	Reference
Pistacia vera	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Plantago lanceolata	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	Pollen viability lost after 1 and 4 hours at low and high RH, respectively	Shivanna and Heslop-Harrison, 1981
Prunus avium	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
P. cerasifera	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen germination and tube length significantly reduced	Torabinejad et al 1998
Prunus cerasus	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen germination and tube length significantly reduced	Torabinejad et al 1998
P. mahaleb	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
P. virginiana	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Pyrus communis	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen germination and tube length significantly reduced	Torabinejad et al 1998
Secale cereale	Exposure of mature dehisced pollen to high and low RH	FCR, pollen germination	Under low RH all viability lost within 2 hours, under high RH 40% viable after 4 hours	Shivanna and Heslop-Harrison, 1981
	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Solanum tuberosum	Effect of high temperature on mature dehisced pollen	Pollen germination	Pollen germination decreased strongly after exposure to high temperature for 30 minutes	Pallais et al 1988
Sorbus aucuparia	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998

Table 3, continued

Species	Treatment/Stress	Analysis	Effect	Reference
Spartium junceum	Exposure of mature dehisced pollen to air	FCR	Viability remains high for 48 hours, then drops quickly	Pacini et al 1997
Spinacia oleracea	Exposure of mature dehisced pollen to air	FCR	Viability drops quickly, all pollen dead after 72 hrs	Nepi et al 2001
Tradescantia blossfeldiana	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
Triticum aestivum	Water stress during pollen development	Grain set	Water stress during meiosis results in 50% decrease in grain set, which is associated with changes in sugars and sugar metabolizing enzymes	Dorion et al 1996
Triticum aestivum	Water stress during pollen development	Light microscopy	Water stress during meiosis results degeneration of meiocytes, loss of polarity, ectopic starch deposition and others	Lalonde et al 1997
Viburnum lantana	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	No significant reduction in pollen germination or tube length	Torabinejad et al 1998
Zea mays	Effect of heat and water stress on pollen viability	Grain set	Heat stress affects pollen viability strongly, water stress not	Schoper et al 1984
	Effect of UV-radiation on pollen development	Pollen germination, electron microscopy	No effect of UV-radtiation during development on pollen viability	Santos et al 1998
	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998
	Exposure of mature dehisced pollen to air	Grain set	Grain set decreased if pollen was exposed to air; after 120 minutes pollen was inviable	Luna et al 2001
	Exposure of mature, dehisced pollen to air	Pollen germination	Exposure to air results in dehydration and increasing loss of viability with time	Aylor 2003, 2004
Zigadenus venenosus	Pollen germination under UV-B radiation	Pollen germination, pollen tube length	Pollen tube length but not pollen germination significantly reduced	Torabinejad et al 1998

The most sensitive period for heat stress was found to be 7-15 days before anthesis, which corresponds to the developmental stage that was very sensitive to drought stress in cereals (Sato et al., 2002). When analyzing concentrations of starch and soluble sugars in tomato anthers and pollen exposed to mild heat stress, it was found that stressed pollen grains did not display the temporal increase in starch concentrations observed in control pollen. In addition, the concentration of soluble sugars in both the anther wall and pollen of stressed plants at dehiscence was lower (Pressman et al., 2002).

In *Arachis hypogaea*, short periods of heat stress during pollen development resulted in drop of fruit set, pollen production and pollen viability. Calculations showed that pollen viability decreased with -1,9% $°C^{-1}$ (Vara Prasad et al., 1999). When a heat-sensitive and a heat-tolerant genotype of *Phaseolus vulgaris* were subjected to mild heat stress during development, pollen viability of the heat-sensitive genotype declined from 80% to below 10% after 10 days of heat treatment. The heat-tolerant genotype, however, still produced 60% viable pollen even after 24 days of stress. Effects of the heat stress on pollen included alterations in the pollen wall and a reduction in the number of pollen grains that adhered to the stigma (Porch and Jahn, 2001).

In conclusion, it appears that heat stress can affect pollen viability, but has limited consequences on mature pollen after dehiscence. In addition, the temperatures required to sort an effect are much higher than usually encountered in natural environments (Rao et al., 1992). Heat stress during pollen development, on the other hand, may have profound consequences for pollen viability.

3.1.3 UV-B radiation

Studies on the effect of UV-B radiation on plant growth and development have mostly been performed in the past 25 years, when it became clear that atmospheric ozone reduction could lead to an increase in UV-B radiation (Caldwell et al., 1998). UV-B radiation (280-320 nm) is normally present in the sunlight that reaches the earth's surface, and it is therefore likely that mechanisms are present in plants to protect them against the damaging effects of UV-B. However, with increased radiation due to decreased ozone these mechanisms could prove insufficient.

On a cellular level, UV-B induced damage may occur in DNA, proteins and lipids. DNA damage can be repaired, and affected proteins and lipids replaced to a certain degree. In addition, some transport processes are perturbed when membranes are

targeted by UV radiation, which in turn may disturb cellular processes (Murphy, 1983). On the whole-plant level increased UV-B radiation may result in reduced growth, but responses differ between species and even between varieties (Caldwell et al., 1998). With respect to reproduction, UV-B radiation may alter timing of flowering and the number of flowers formed. The potential damage by UV-B radiation to the pollen during the transfer from the anther to the stigma may be reduced by flavonoids present in the pollen wall (Stapleton and Walbot, 1994). In addition, dispersal of pollen clustered in some way may prevent the inner pollen grains from radiation damage (Dafni and Firmage, 2000).

In an early analysis of the effect of UV-B radiation on germination, pollen grains were exposed to radiation levels similar to those at either temperate latitude or equatorial alpine locations (Flint and Caldwell, 1984). In three of four species analyzed, higher radiation levels caused partial inhibition of germination (33-52%), but at low levels no effect was observed. A more extensive analysis included 34 species that were treated with two levels of UV-B radiation (Torabinejad et al., 1998). Species responded differently to the treatments. Significant reductions in pollen germination rates were found for 5 species only, while more than 50% showed a significant reduction in pollen tube lengths. However, some species showed an increase in germination rates or pollen tube lengths (Torabinejad et al., 1998). Although the differences were small, it appeared that trinucleate pollen and pollen of monocotyledonous plants were more sensitive to UV-B radiation.

The effect of UV-B radiation on reproduction processes in Brassica has been analyzed in more detail than for most other species (Demchik and Day, 1996; Feldhim and Conner, 1996; Conner and Zangori, 1997). For *B. rapa* it was shown that UV-B radiation decreased pollen production and pollen germination rates and viability, but the effect was less prominent in plants continuously exposed to increased UV-B levels, indicating that protective mechanisms may be induced. However, when UV-B radiated pollen was used in pollinations and seed set was analyzed, no differences could be found (Demchik and Day, 1996). Furthermore, analysis of offspring produced in these experiments indicated no effects of UV-B radiation (Feldhim and Conner, 1996). Finally, in a garden experiment it was found that increased UV-B radiation did not affect pollen or seed production of *B. nigra* or *B. rapa* plants.

These results indicate that increased UV-B radiation has negative effects on pollen performance, but that seed set in general will probably not be affected. One explanation for this observation may be that pollen tubes growing in the pistil are

shielded from most radiation, whereas in most *in vitro* experiments radiation on pollen continued during growth. Another possibility is that hand pollination caused an overabundance of pollen on the stigma. However, detailed data on the effect of UV-B radiation on seed set is limited to a few species, and it is possible that in some species seed set may be affected.

3.1.4 Transport

One of the crucial events in the fertilization process of higher plants is the transfer of pollen grains from the anther to the stigma, often of another flower. In the case of wind pollinators, the transport of pollen trough the atmosphere will probably have similar effects as exposure to the environment, which was shown to rapidly decrease viability of maize pollen (Luna et al., 2001; Aylor, 2003, 2004). In the case of pollination by insects or other animals, the effect on viability may depend strongly on the type of pollinator. In nature, many invertebrate and vertebrate species can and will act as pollinator. However, honey bees are often seen as the most efficient pollinators, possibly because of their frequent use in agriculture and because of their ability to adapt to many environments around the world. As literature dealing with the effect on pollen of transport by insects other than bees is extremely rare, we will focus here on bees only.

Bees collect pollen which serves as source of proteins, fatty substances, minerals and vitamins. Pollen is therefore indispensable for the bees' survival; pollination of flowers occurs only as a side effect during the collection of pollen. Pollen is collected with the bees' mouth parts, is moistened and subsequently kept on the posterior pair of legs. However, numerous pollen grains stick to body hairs, and may remain dry. Once returned to the hive, the bee unloads her pollen and grooms herself. Despite this, 10000-25000 pollen grains remain on the bees' body, which was reported to be more than of any other hairy insect (Lukoschas, 1957). Very few reports exist that consider the viability of pollen grains present on the body hair of bees. It has been reported that bees carrying viable Brassica pollen could be found emerging from the hive (Eastham and Sweet, 2002). In addition, for Cantaloupe (*Cucumis melo*) it was reported that viability of pollen present on the bee was lower (46–28%) than pollen of the same age present on the anther (>80%) (Vaissiere et al, 1996). Because the age of pollen was never more than 4 hours, it is unclear when the pollen looses viability completely. However, considering the rapid decline it seems unlikely that pollen would still be viable after a night in the hive.

The bees' choice for a specific foraging site is determined by various factors. Bees tend to forage repeatedly on flowers of the same species, if possible close to the hive. Only when the foraging area becomes less attractive, bees will find a new food source by increasing the flight distance (Graham, 1992). Factors negatively affecting the attractiveness of flowers include absence of pollen, cessation of nectar and scent production, changes in color, wilting, permanent flower closure and petal drop (Delaplane and Mayer, 2000).

Bees may carry pollen over considerable distances and have been found foraging in fields more than 4 km away from the hive (Eastham and Sweet, 2002). Therefore, if in-hive pollen transfer between bees occurs, the pollen may be transported over distances close to 10 km. Although many species, like Brassica, are generally thought to be mainly pollinated by bees, spreading of pollen by wind may also be a factor. Timmons et al. found that at 350 m from a Brassica source field, the percentage of Brassica pollen found in the air was 10% of that at close distance to the field. In addition, background concentrations of Brassica pollen could be detected at larger distances (Timmons et al., 1995). Despite these findings, it remains unclear if wind dispersed pollen are still viable and capable of achieving fertilization. Some evidence has been found in favor of this possibility, but in these cases pollination by insects could never be ruled out (Eastham and Sweet, 2002; Ramsay, 2003, Timmons et al 1995). For other species, dispersal of pollen by wind may occur over varying distances. For example, sugar beet pollen was found more than 1000 m from the source, but potato, maize and wheat pollen are not dispersed over distances more than 100 m from the source (Eastham and Sweet, 2002).

3.1.5 Other factors affecting pollen viability

The environmental factors listed above affect the viability of pollen of a large number of species. However, few additional factors are known to influence pollen viability. For example, elevated CO_2 levels protect pollen viability against heat stress during development (Aloni et al., 2001). As heat stress and also drought stress during pollen development are associated with changes in sugar metabolism (Saini, 1997; Pressman et al., 2002), it is possible that the elevated CO_2 causes increases the assimilate availability.

Pollen viability is not only influenced by environmental factors. Different genotypes of *Picea abies* were shown to vary with respect to pollen viability (Nikkanen et

al., 2000), and in *Prunus avium* both the genotype of the pollen and the pistil affected pollen tube growth (Hormaza and Herrero, 1999).

3.2 Concluding remarks

From reports on the occurrence of hybrids it can be concluded that, in general, pollen are viable under a large variety of circumstances. For example, it was shown that throughout the world 12 of the 13 most important domesticated crops hybridized with wild related species (Ellstrand et al., 1999). Because transgenic crops generally do not behave differently from non-transgenic, it may be expected that pollen viability will not prevent hybrid formation between transgenic plants and wild species. However, the rates of hybrid formation are dependent on various factors, and vary strongly from place to place and between species (de Vries et al., 1992; Raybould and Gray, 1993; Ellstrand et al., 1999). One of the factors contributing to this difference could be the viability of pollen.

Most recent literature on pollen viability deals with the effect of water balance, temperature stress and UV-B radiation. As the experimental conditions vary strongly with respect to treatments and measurements of pollen viability, it is difficult to directly compare them. However, it appears that these factors affect pollen viability of many species to a certain degree, but that the exact effect is strongly species dependent. This is especially true for dehydration stress. In many partially hydrated pollen, dehydration leads to rapid loss of viability. However, adaptations can be found in some species with partially hydrated pollen that prevent hydration or the damage caused by it. Heat stress has less effect on mature dehisced pollen grains of many species, with the exception of a few. UV-B radiation, by contrast, does affect pollen tube growth of many species, but for most species it is unclear if this has an effect on seed set. Together, it appears that pollen is affected by factors that are encountered in the environment, but that viability is not completely lost. Obviously, considering the function of pollen, this is not surprising.

Interestingly, pollen viability is not only influenced by the environment after dehiscence, but also during development in the anther in a number of species. The stresses that lead to a reduction in pollen viability in these species are different, but the effect on pollen and pollen viability are surprisingly similar and involve, amongst others, changes in sugar and starch concentrations and in the activities of enzymes involved in sugar-starch metabolism. In this light, it is notable that in some Gramineae species presence or absence of sucrose in mature pollen is associated with the prevention of

dehydration damage (Hoekstra et al., 1989). Unfortunately, in general the effects of the changes in sugar-starch mechanism on the pollen and pollen dehydration in the anther and rehydration on the stigma are unknown.

In conclusion, pollen viability may play a critical role in the efficiency of hybrid formation. However, since pollen of different species and sometimes even varieties show different responses to environmental factors, it is not feasible with the current knowledge to predict the contribution of pollen in hybrid formation for most species.

Part 2 Pollen Viability Experiments

Abstract

For sexual plant reproduction to occur, viable pollen grains must be deposited onto a compatible stigma. The efficiency of pollen transfer and viability of the pollen grains therefore determine in part the reproductive success of a species. In recent years various transgenic crops have been introduced into the environment. The use of such crops may be beneficial for various reasons, but has also raised concerns with respect to the possible escape of transgenes from these crops and introgression into non-transgenic crops or related wild populations. One of the ways that transgene escape can occur is through pollen-mediated sexual reproduction and pollen viability may therefore be a crucial factor in determining the frequency of transgene escape. In order to better estimate the possible role of pollen viability in transgene escape, we analyzed the viability of pollen grains of three different crops commercially grown in the Netherlands. Our results show that pollen grains lose viability with different kinetics, even for pollen grains that share physiological characteristics. In addition, it is found that pollen viability in the field is highly variable, indicating that differences in microenvironment may have a profound effect on pollen viability.

4 Introduction

The use of transgenic crops in agriculture may be advantageous for various reasons. For example, yields can increase and the amounts of herbicides can be reduced. However, the use of transgenic plants is accompanied by the possibility of transgene escape into wild populations of identical or related species, which can have various ecological consequences (Ellstrand, 2001). Transgene escape can occur in various ways. In addition, the frequency of transgene escape depends on various factors, including favorable geographical conditions, the presence of related species, the efficiency of pollen transfer and fitness of the formed seed and hybrid. Recently, considerable attention was given to a number of these factors (de Vries et al., 1992; Eijlander and Stiekema, 1994; Groot et al., 2003; Ramsay, 2003).

In addition to the previously mentioned factors, pollen viability may in part also determine the frequency with which transgene escape occurs. Traditionally, research on pollen viability focused on factors that could preserve viability (Stanley and Linskens, 1974). Only recently, more research has been devoted to the factors that decrease the viability of pollen grains and may be encountered in the field, mainly during the period from dehiscence until pollen tube growth in the style. The rate of pollen viability loss strongly depends on the species and type of pollen grain. Generally, pollen grains that are partly hydrated at dehiscence (water content > 30%; (Nepi et al., 2001)), are very sensitive to water loss and loose viability rapidly. Maize pollen grains, for example, retain viability for 1 to 4 hours after dehiscence, depending on the relative humidity (Luna et al., 2001; Aylor, 2003, 2004). Generally, the exposure to dehydrating conditions has adverse effects on the viability of partially hydrated pollen grains, but partially dehydrated pollen grains may also suffer from such conditions (Shivanna and Heslop-Harrison, 1981; Nepi et al., 2001). In addition to water loss, pollen grains may also be sensitive to temperature stress and UV-B radiation (Pallais et al., 1988; Rao et al., 1992; Torabinejad et al., 1998; Pressman et al., 2002). However, these effects appear smaller and are even more species-dependent than the effect of water loss.

Pollen viability is not only affected by environmental factors during the transport from the anther to the stigma, but pollen development inside the anther may also be disturbed by the same factors. Drought stress during pollen development of cereals, for example, specifically reduces male reproductive performance (Saini, 1997). Likewise, temperature stress during floral development may affect reproductive performance. In

tomato, for example, a slight rise in average temperature drastically reduces fruit formation and seed set (Peet et al., 1998). The effect of heat stress during development includes a reduction in the number of pollen grains released, but also a 75% decrease in pollen viability (Sato et al., 2000). Correlations between heat stress and loss of pollen viability have been found for various plants, indicating that it may be a rather general response (Vara Prasad et al., 1999; Porch and Jahn, 2001).

Many of the reports on pollen viability merely consider the effect of a single factor, often under controlled experimental regimes. Obviously, such experiments allow detailed analysis of the effect of the variable factor on viability. However, pollen grains in the field may be subjected to multiple stresses simultaneously, and, moreover, stresses may be present during development of the pollen in the anther. Therefore, the loss of viability as determined in a controlled setup does not necessarily correlate to the loss of pollen viability in the environment. Moreover, such results may not be suited to predict the contribution of pollen viability on transgene escape. Only in a few cases, pollen viability has been determined in the field or after exposure to the environment (Conner and Zangori, 1997; Aylor, 2003, 2004).

As pointed out above, pollen viability can be one of the factors that determine the rate of outcrossing and transgene escape. In general, it may be expected that pollen which is highly viable for a long period of time may contribute significantly to transgene escape. Pollen that remains viable for short periods of time, on the other hand, will have a smaller contribution to transgene escape. To allow an estimation of the possible contribution of pollen in this process, we have analyzed the effect of exposure to the environment on pollen viability for three crop plants growing in the Netherlands, Brassica (*Brassica napus*), strawberry (*Fragaria annanassa*) and wheat (*Triticum aestivum*). These species are commercially grown in the Netherlands and are interesting for biotechnology companies, with various transgenic varieties existing for all species (Eastham and Sweet, 2002). *Brassica* and strawberry have partially dehydrated pollen and are mainly pollinated by bees, whereas wheat has hydrated pollen and is a wind pollinated species.

5 Material and methods

5.1 Pollen collection and germination

Brassica. Brassica (*Brassica napus*) pollen samples were collected in Nieuwolda (Groningen, the Netherlands), on April 26 and 29, May 7 and May 13, 2004. In addition, Brassica pollen samples were collected on May 4 near Heteren, Zetten and Druten (Gelderland, the Netherlands). Weather conditions during all sampling dates were calm and dry, with the exception of May 7, when approximately 5 mm rain fell. Brassica flowers were grouped in four stages. Flowers with dehisced anthers and a partially opened corolla were designated as stage 1. Stage 2 flowers were approximately 24 hours older than stage 1. In these flowers, the corolla had fully opened. In stage 3 flowers, which were approximately 48 hours older than stage 1, the corolla ends were located below the level of attachment to the flower. Finally, stage 4 flowers were approximately 72 hours older than stage 1, and in these flowers the corolla started to senesce. In addition, the initiation of fruit formation was visible in flowers of this stage.

Because we wanted to determine viability rates of pollen from individual flowers, two anthers of a single flower were dropped in 100µl pollen germination medium (PGM: 0.01% H_3BO_3, 0.07% $CaCl_2.2H_2O$, 3.0% PEG 4000 and 20% sucrose). The pollen grains were allowed to germinate for at least 2 hours before the samples were fixed by the addition of 0.8% formaldehyde to the germination medium. The pollen samples were observed and photographed using a Leitz Orthoplan (Leica Microsystems GmbH, Wetzlar, Germany) microscope equipped with a Color Coolsnap digital camera (Roper Scientific, Tucson, AZ) and the MetaVue software (Universal Imaging Corporation, West Chester, PA). Germination frequencies were established by counting germinated and non-germinated pollen grains. A pollen grain was scored as germinated if a pollen tube was present that was at least the size of the pollen diameter.

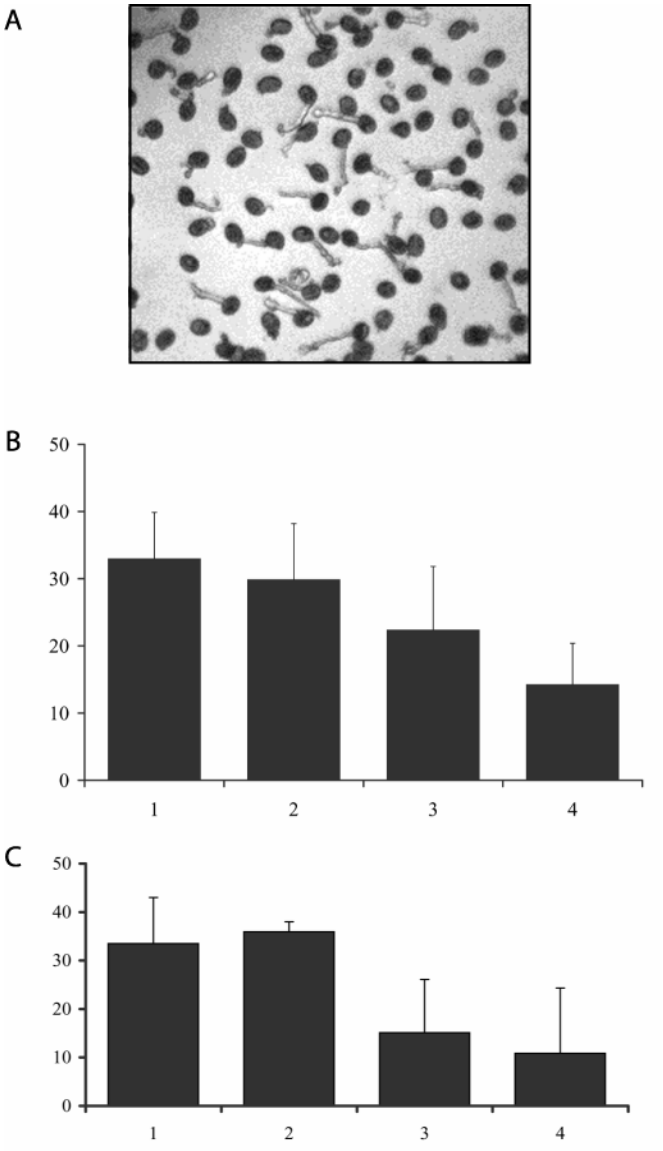

Figure 1. Brassica pollen grains collected and germinated in vitro in the field A) Picture taken with light microscope showing a typical sample of germinating pollen from a stage 1 flower. B) Average germination percentages of all Brassica pollen samples collected in dry weather. C) Average germination percentages of Brassica pollen samples collected in wet weather. Numbers indicate flower stage, error bars represent standard deviation.

Figure 1A shows an example of germinating Brassica pollen grains. *Strawberry.* Strawberry pollen samples were collected in a field near Elst (Gelderland, the Netherlands) on June 15 and 17. The strawberry plants growing at this site were test

crosses between various late flowering strawberry races. Six anthers from a single young flower were placed two by two in three wells of a microtiter plate, and exposed to the environment. On June 15, pollen samples were allowed to germinate by the addition of 100μl PGM after 0 or 2 hours of exposure to the environment. On June 17, pollen samples were exposed for 2 or 6 hours. On both days, it was dry during pollen collection and exposure, although it some rain had fallen before pollen collection on June 17.Pollen germination rates were established as the Brassica samples. *Wheat.* Wheat pollen samples were collected in a field in Lent (Gelderland, the Netherlands) on June 10 and June 15. On both days, weather conditions were dry and calm. Various florets of a spikelet were analyzed in order to find the developmental stage that would allow pollen collection. The presence of pollen in the microtiter well was visually confirmed. However, because of the apparent lack of correlation between floret appearance and the presence of pollen, the actual age of the pollen grains was unknown. Collected pollen grains were germinated in PGM either immediately after collection, or after 15 or 30 minutes exposure to the atmosphere. Pollen germination rates were established as the Brassica samples.

5.2 Scanning Electron Microscopy

For the *in vivo* germination, young flower buds with undehisced anthers were emasculated. Next, the emasculated flower buds were pollinated with pollen grains of flowers at stages 1-4 by brushing anthers of these flowers onto the stigmas of the emasculated flowers. The pollinated flowers were then removed from the plant and placed in Eppendorf tubes containing water. After 3 hours incubation to allow pollen adhesion, hydration and germination, the stigmas were removed, placed onto sample carriers and frozen in liquid nitrogen. Scanning Electron Microscopy was carried using a JEOL JSM T300 Scanning Electron Microscope (working distance 12mm, 3.0 kV). Digital pictures were made and used to determine germination frequencies. An example of *in vivo* germinating pollen is shown in Figure 2A.

6 Results

6.1 In vitro germination of Brassica pollen grains

To determine the viability of Brassica pollen grains present on flowers of various ages, we collected anthers of such flowers on various locations in the Netherlands (see Materials and methods) and allowed the pollen grains of these anthers to germinate *in vitro*. The results, presented in figure 1, show that the average germination percentages of Brassica pollen grains decreased from approximately 35% in fresh flowers of stage 1 flowers to 15% in flowers of stage 4, which are approximately 72 hours older than stage 1 (see Materials and methods for a description of flower stages). The germination rates of the different pollen samples varied considerably between flowers. For pollen collected from stage 1 flowers, germination percentages ranged from 44% to 13%, although only 3 out of 18 samples showed less than 25% germination. Germination percentages of pollen collected from stage 2 flowers ranged from 46% to 10%. For pollen collected from stage 3 flowers, germination percentages lay between 38% and 3%. Finally, pollen collected from stage 4 flowers showed germination frequencies from 30% to 0%, with 14 out of 19 samples being lower than 20%. We did not find any correlation between pollen germination rates and environmental factors or specific locations (supplements), although it was observed that generally the number of pollen grains present in stage 1 and 2 flowers was higher than in older flowers. On one occasion pollen grains were collected shortly after rain, and the germination rates of these samples was determined. The results indicated that germination rates were not different from pollen collected in dry weather; for pollen collected from stage 1 flowers, for example, germination percentages between 25% and 44% were found. However, the number of pollen grains per anther after rain was much lower when compared to dry weather conditions, especially for stage 1 and stage 2 anthers.

Interestingly, if anthers with pollen were taken to the laboratory and allowed to germinate after 3 hours of collection, germination rates were severely reduced (data not shown).

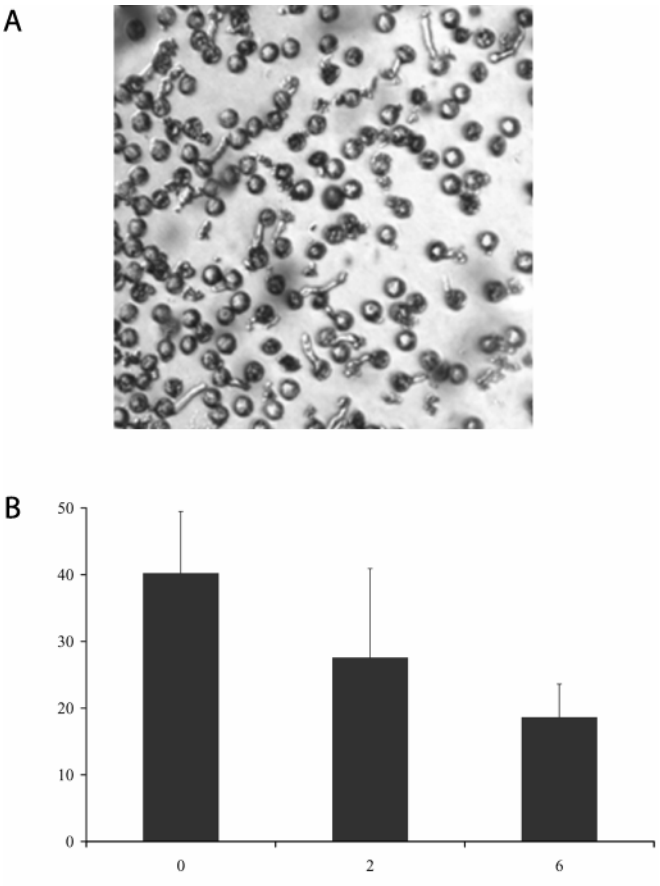

Figure 2. In vitro germination of strawberry pollen grains. A) Picture taken with light microscope showing a typical sample of germinating fresh strawberry pollen. B) Average germination percentages of all strawberry pollen samples. Exposure time to the environment is indicated on the x-axis, error bars represent standard deviation.

6.2 In vivo germination of Brassica pollen grains

It is known that *in vitro* germination of pollen grains can lead to an underestimation of pollen viability rates. Therefore, in addition to the relatively quick and easy *in vitro* germination procedures, we analyzed pollen germination *in vivo*. To this end, we collected anthers as described above and used them to pollinate emasculated flowers. After at least two hours incubation to allow pollen adhesion, hydration and germination, the stigmas were isolated, attached to Scanning Electron Microscope (SEM) sample carriers and frozen in liquid nitrogen. The stigmas were observed with SEM and the pollen germination percentages were determined. The results showed that the

germination percentages ranged from 75% in stage 1 flowers to 15% in stage 3 flowers (Figure 2). Interestingly, the differences in germination percentages between the samples were much lower than that observed during the *in vitro* germination experiments.

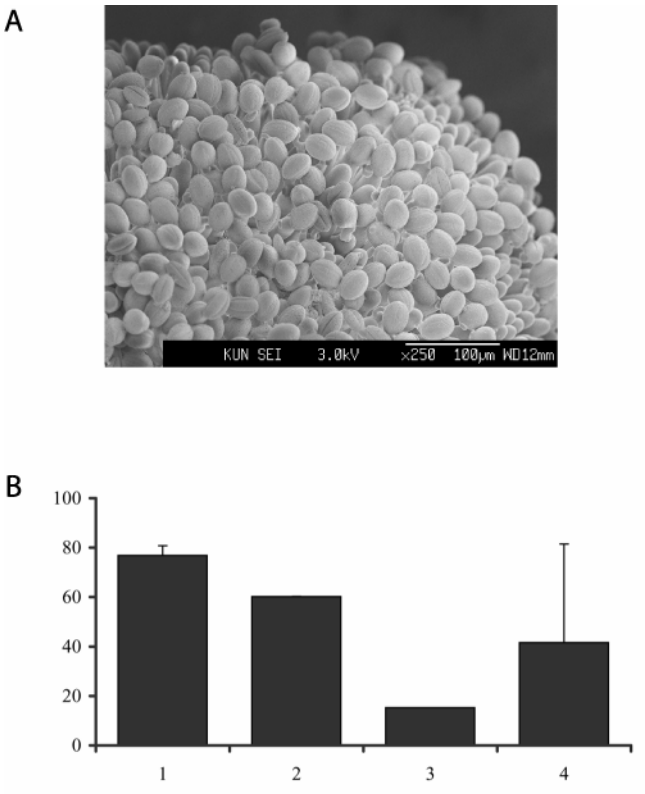

Figure 3. Brassica pollen grains collected and germinated in vivo in the field. A) Picture taken with a scanning electron microscope showing a typical example of germinating pollen on the stigma. B) Average germination percentages of Brassica pollen on the stigma. Numbers indicate flower stage, error bars represent standard deviation.

6.3 In vitro germination of strawberry pollen grains

To analyze the effect of exposure to the environment on the viability of strawberry pollen grains, we exposed strawberry pollen grains to the environment and determined *in vitro* germination rates. To this end, we collected freshly dehisced anthers of strawberry flowers and placed them in a microtitter plate. At various time points pollen germination

medium was added to the anthers and germination rates were determined. The results, shown in figure 3, show that the average germination percentage of fresh strawberry pollen is approximately 40%. Exposure to the environment results in a decrease of germination efficiency to approximately 30% after 2 hours and 20% after 6 hours. As with the Brassica pollen samples, the germination rates varied considerably between different strawberry lines (Supplements). Germination percentages of fresh pollen, for example, ranged from 50% to 30%, whereas after six hours exposure to the environment they ranged from 23% to 12%.

6.4 In vitro germination of wheat pollen grains

To analyze the effect of exposure to the environment on pollen that is partially hydrated at dehiscence, we set to determine the *in vitro* germination rates of wheat pollen grains. Because it is known that partially hydrated pollen generally remains viable for short periods, we used short exposure times of 0, 15 or 30 minutes. Collecting fresh, just dehisced anthers and pollen was complicated by the absence of clear morphological markers indicating the time of anther dehiscence. As a consequence, although the presence of pollen grains was visually confirmed, it was impossible to determine the exact age of the pollen grains. This difficulty was reflected in the results, which showed that germination percentages were highly variable, ranging from 0% to 60% (see supplement). However, the effect of exposure time of the pollen grains to the environment was not reflected in the germination percentages, possibly because the actual age of the pollen at the start of the experiment was unknown. Therefore, using *in vitro* germination was not successful in this species, and the experiments were discontinued.

7 Discussion

The introduction of transgenic crops is accompanied by the possibility of transgene introgression into related crop or wild species. The frequency of occurrence of such transgene escape depends on many variables, and is therefore hard to predict. One of the factors that may determine the rate of transgene escape is pollen viability. It has been shown that pollen viability may be reduced by factors that act during development or during the transport from anther to stigma. However, limited data is present on pollen viability rates in the field and of the exposure of the pollen to the multiple but mild stresses that are likely to be encountered in the field. Therefore, we have analyzed pollen viability rates of Brassica, strawberry and wheat pollen that developed in the field, by measuring germination rates *in vitro*.

Pollen grains can be considered viable if they are able to achieve fertilization in a natural setting. Therefore, analyzing pollen viability would ideally involve analyzing seed set after natural pollination in the field. However, fertilization is not only dependent on pollen viability, but on other factors too, such as pollen dispersal and ovule development in the mother plant. Various methods exist to determine pollen viability that bypass the need for analyzing seed set. Some of these methods include enzymatic stains that test the integrity of the plasma membrane and/or the activity of cytoplasmic enzymes. These so-called vital stains have various advantages, but generally over-estimate pollen viability. Moreover, the fluorochromatic reaction, the most frequently used vital stain, requires a fluorescence microscope and is therefore not suited for evaluating pollen viability in the field. In our experiments, we primarily used *in vitro* germination to evaluate pollen viability. Pollen grains were germinated immediately after collection from flowers in the field, without the need to transport the pollen grains to the laboratory. Thus, in this case, germination percentages may accurately reflect pollen viability in the field. However, *in vitro* germination conditions are not optimal for pollen tube formation in many species, and therefore germination percentages will usually be lower than the actual pollen viability rates. This possibility can explain the higher germination percentages we observed after *in vivo* germination of Brassica pollen.

Pollen viability rates are determined by factors such as water balance, temperature stress and UV-B radiation. The effect of these factors on viability varies with species, and various adaptations that reduce damage can be found in pollen grains. Such adaptations may include the dehydrated state of mature pollen grains, the

presence of specific sugars in the pollen cytoplasm or various structural adaptations. The Brassica and strawberry pollen that we have analyzed are both partly dehydrated, and are both pollinated by bees. However, decreases in viability rates occur much faster for strawberry than for Brassica pollen. In similar *in vitro* germination experiments Brassica pollen viability decreases from 35% to 20% in 72 hours, whereas a similar decrease in viability occurs within 6 hours for strawberry pollen. Possible explanations for this difference include the presence of different sugars in the pollen cytoplasm, or different hydration states of the pollen at maturity (Pacini, 2000). However, it is also possible that differences in pollen presentation or pollen packaging are involved. Brassica pollen grains are encapsulated in the flower corolla at dehiscence, which may provide some protection from the environment.

Our results indicate that the viability of strawberry pollen decreases faster over time than that of Brassica. However, the variability in the germination percentages of pollen samples of all species investigated was surprisingly high. In some cases, the viability of pollen samples collected on the same day and place, from flowers of the same stage, could even range from 0% to 40%. For the wheat pollen samples, we believe that the large variability is caused by a combination of the short time wheat pollen remains viable (Poehlman, 1987) and the difficulties in obtaining fresh pollen. However, this does not apply to the Brassica and strawberry pollen samples. For these plants we believe that the large differences between pollen samples reflect true variance in the viability of pollen grains in the field. Pollen viability has been shown to be determined by various factors that may be present in the field to different extents. Such factors, for example temperature and relative humidity, may seem uniform field scale, but could vary considerably in the microenvironment of the flower and anther. In addition, bees or other animals may visit some flowers more frequently than others. It is not known whether visits by bees have an effect on the viability of pollen that remain on the anther. However, it is possible that the viability of the pollen grains remaining on the anther is affected at the time bees moisten the pollen for collection (Graham, 1992).

Considering the role of pollen in outcrossing and transgene escape it must be stressed that these mechanisms depend on many variable factors, of which the viability of pollen is one. Knowing pollen viability rates could result in an estimation of outcrossing risk, assuming that pollen which remains viable for longer periods of time would have more chance on outcrossing. However, this assumption has not been experimentally verified. Therefore, predicting outcrossing rates on the basis of pollen viability alone is not feasible. Such predictions should only be made after experiments

that directly measure outcrossing, for example by using bait plants and sensitive techniques to detect outcrossing events. Such experiments have been performed on a large scale for Brassica (Ramsay, 2003).

8 General conclusions

Part 1: Literature review

- Factors that affect pollen viability may do so during the period after anther dehiscence, but also during pollen development in the anther
- Drought and heat stress are the most common factors that affect pollen viability during its development in the anther
- Dehydration, heat stress and UV-B radiation are the most common factors that affect pollen viability after release from the anther
- Rates of viability loss caused by any of the factors is highly species specific
- Very little information is available on the effect of multiple stresses, which are likely to occur in the field

Part 2: Viability experiments

- For two out of three species investigated (Brassica and strawberry), pollen germination experiments proved to be a suitable tool to analyze pollen viability
- Nevertheless, pollen germination underestimates actual pollen viability rates
- Viability rates in the field are rather variable from flower to flower, for both Brassica and strawberry
- Pollen viability loss have different kinetics in Brassica and strawberry

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