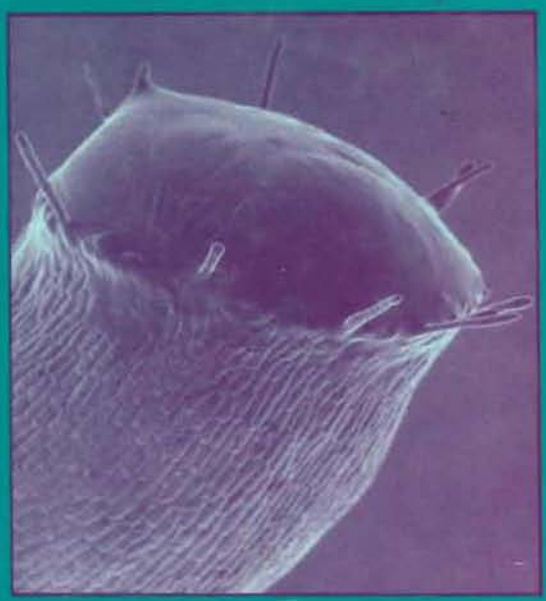
M. Cresti
A. Tiezzi
Editors

# Sexual Plant Reproduction



Springer-Verlag

# Cytological Techniques to Assess Pollen Quality

J.S. Heslop-Harrison
Karyobiology Group
Department of Cell Biology
JI Centre for Plant Science
Norwich NR4 7UJ England

## The Aim of Assessing Pollen Quality

Viable pollen is pollen that is competent to deliver two male gametes to the embryo sac. Methods to assess pollen quality aim to find the capacity of pollen - individual grains or members of a population - to deliver gametes. Pollen quality is normally measured 1) by scoring seed set in plants fertilized with a particular pollen sample, 2) by cytochemical staining of the grains, or 3) by looking at germination of the pollen *in vitro* or on styles. Table 1 includes the sequence of events that a viable pollen grain must complete. A failure at any stage gives male sterility, since the pollen is then unable to deliver the gametes.

Table 1. The sequence of events in an angiosperm pollen grain's formation and life.

A pollen mother cell must:
- go through meiosis,
- divide and
- differentiate into pollen grains.

A pollen grain must:
- dehisce, and perhaps need a maturation period,
- attach to a stigma,
- hydrate,
- germinate and
- produce a pollen tube.

A pollen tube must:
- penetrate the stigma,
- enter the pollen tube transmitting tract,
- the generative nucleus must divide (in the 70% of families where the pollen is binucleate when shed),
- the tube must grow through the style to the ovule and
- enter the embyro sac and release the gametes.

## The Need to Assess Pollen Quality

Pollen quality must be assessed to find plant fertility, to monitor pollen state during storage, in ecological or taxonomic studies and in research on pollen biochemistry, genetics and stigma interactions, incompatibility systems and fertilization (see Stanley and Linskens 1974; Heslop-Harrison, Heslop-Harrison and Shivanna 1984). The tests described here allow measurement of pollen viability by examining and measuring cellular features characteristic of living cells, and they indicate reasons for inviability. Knowledge the nature of inviability can often enable manipulation to overcome the barrier (see, e.g., van Tuly, this volume) although factors influencing pollen viability have proved elusive to understand and control (Knox, Williams and Dumas 1986).

Pollen viability is important in agriculture and for plant breeders since pollen must be viable at the time of pollination for seed (or fruit) set to occur. In apple production, boron deficiency can cause low pollen germinability, and hence poor fruit set; application of boron can correct the problem. Recently, a new variety of wheat in the UK, Moulin, failed in its first year of release because a period of cool, dull and wet weather during microsporogenesis caused poor pollen production. In other contexts, pollen may be stored for germplasm conservation, to make hybrids between plants that flower at different times or places, or for later use in hybridization programmes, and the quality must be monitored. Many economically important plants are propagated vegetatively by bulbs, ramets, tubers or cuttings - potatoes, rubber trees, and lilies are examples. But sexual hybridization is still required for breeding new varieties. Studies of pollen quality are particularly important because the crops often show low fertility and are hence difficult to intercross. Plant breeders use wide hybrids between different species or even genera to transfer alien genes into major crop plants: in wheat, such chromosome engineering is now the only method of genetic manipulation with new genes (see, e.g., Gupta and Tsuchiya 1991). However, the barriers to fertility both in making the hybrids and in the resultant hybrids usually require careful investigation.

## Pollen State

Pollen is living, and, like any living organism, its behaviour and survival are influenced by both environment and genotype. Therefore, pollen



quality must be measured under defined conditions, and thought about experimental and plant growth conditions is always required. In experimental treatments, possible variables must be carefully considered, and controls used. Pollen production and quality vary from hour to hour, day to day and season to season. Many species have strong diurnal rhythms: in the summer, anther dehiscence may occur at 5 am, and pollen collected 12 h later may be inviable. The nutrition of the parent plant can also have large effects on pollen viability (and may affect results from the *in vitro* germination tests discussed below more than growth on the stigma).

Some pollen will show no viability in tests unless it is correctly preconditioned by, for example, leaving in a humid atmosphere before testing. For most species, the requirements for such preconditioning or post-maturation are not established, so cytological methods to estimate viability may give an unrealistic estimate of quality.

## Safety of Tests

Pollen is a severe allergen, and hence is a hazardous substance in the laboratory. Plants with wind dispersed pollen should be kept in enclosed areas and a dust mask used if you are working closely with such plants. Wash away spilt pollen from working areas and from your hands and face. Allergies can be induced by repeated contact with pollen, so insensitive people must be particularly careful.

Most of the chemicals used in pollen tests are of low hazard, and good laboratory practice (GLP) can be used to handle them at the low concentrations involved in the tests, although fluorescein diacetate and DAPI are toxic, so they should be dispensed in a fume cupboard before dilution. The chemicals used are not environmental hazards in small quantities and contain no heavy metals, so they can generally be disposed of to mains drainage or with domestic refuse.

## Microscopy

Light microscopy is one of the most important techniques for examining pollen quality since it allows clear observation of pollen morphology. It is useful to examine pollen before carrying out any of the other tests described here to monitor its condition. Staining methods are discussed below, but direct examination of anthers and pollen will confirm that

grains are present in anthers, and show undifferentiated or grossly shrunken grains. Most pollen samples will have a few such inviable grains, but the presence of many aborted grains indicates substantial infertility. The causes may include genetic sterility of hybrids, or severe environmental stress. Since microscopy is quick, good pollen can be immediately used for pollinations, and field examination is possible.

Pollen can be observed as shed from the anther under a dissecting microscope, dry at low power under a transmitted light microscope, or at higher powers when mounted in a medium of suitable osmotic strength (typically 10-20% sucrose to prevent bursting), or suspended in an organic solvent such as acetone. Electron microscopy, although not a method for "scoring" pollen viability may be vital to investigate reasons for pollen sterility (e.g. Mogenson and Ladyman 1989).

**Seed Set**

Measurement of seed set examines the capability of pollen to fertilize a given plant, and in some species provides a standard against which to test other methods. Every step listed in table 1 must be successful, so the seed set test involves assessment of not only the state of the pollen, but also that of the female parent, and of the compatibility of the pollen with the female parent.

Clean (unpollinated) receptive, female stigmata are pollinated lightly with the test pollen. Flowers with mature, unpollinated stigmata can be used, but some species require female inflorescences to be emasculated or bagged to prevent pollination until the stigmata are fully receptive. The pollen for testing is applied to the stigma with some care: too much pollen may prevent hydration, while use of too little may be unsuccessful because of the mentor effect, where several grains must be together before they will germinate and grow successfully. In some species, rough handling of the stigma will cause it to become unreceptive, while in other species, such as *Vicia faba*, scarification is essential (see chapter on The Stigma). The success of pollination can be scored after a few days by looking for fertilized ovaries, and seeds can be counted after ripening, usually 1 to 3 months after pollination.

The test uses the pollen for fertilization, and hence the genes from the pollen can be analysed in later generations. Very low



frequencies of viable pollen may be detected, and field scale tests may be carried out. The test examines every step of the compatibility between the pollen and female parent, and can detect nuclear damage to the pollen (from radiation). However, it is slow, percentages of good pollen grains cannot be obtained easily, and female fertility and compatibility are also tested. Contamination, parthenocarpy and parthenogenesis may be problems, so genetic markers may be required to confirm the male parent of seeds. Finally, artificial pollination is not routinely successful for some plants, including economically important crops - rubber and perennial *Arachis* (Lu, Mayer & Pickersgill 1990).

**Pollen 'Staining' Tests**

*Fluorochromatic reaction (FCR test)*

This is among the best and most widely used tests of pollen viability (Heslop-Harrison, Heslop-Harrison and Shivanna 1984). It tests principally the integrity of the vegetative cell plasma membrane, and relies on the presence of a non-specific esterase in the pollen cytoplasm. Membranes are permeable to the non-fluorescent, polar molecule, fluorescein diacetate, so it can enter the pollen grain. Active esterases within viable grains cleave the acetate residues, leaving the fluorescent molecule, fluorescein, that accumulates in the grain if the membrane is intact. Hence, only grains with intact membranes and active esterases fluoresce. The test is extremely sensitive, reliable, simple to use, takes only a few minutes to perform, and many papers indicate the results strongly correlate with other pollen tests, including germination and seed set. Some false negative results occur, particularly for pollens that have an ineffective, porous plasma membrane prior to hydration. Therefore it is also important that the pollen is correctly pre-hydrated, since the treatment does not reproduce the slow hydration that occurs on a stigma. The development of the fluorescence may take 30 min or more, but rapid scoring is sometimes needed since the fluorescein may leak from the pollen.

In use, the pollen sample is dispersed on a slide in fluorescein diacetate in a sucrose medium (2 mg/ml fluorescein diacetate in acetone; add dropwise to 2 ml 10-20% sucrose with 1-3 mM $H_3BO_3$ and $Ca(NO_3)_2$, adjusted to minimize plasmolysis and bursting, until the solution is just persistently cloudy). Under an epifluorescence

microscope, 5-30 minutes after staining, viable grains fluoresce a bright yellow-green colour under UV illumination, while inviable grains are only weakly stained. It is easy to score the percentage of viable grains, although is some species the test may not give clear differentiation.

*Acetocarmine and DAPI*

These stain the pollen grain nuclei, and acetocarmine weakly stains the cytoplasm, and gives good contrast between the grain and surrounding medium. Any nuclear aberrations are clearly visible. The methods are also convenient for examination of archesporial cell development from premeiotic stages to mature pollen.

Fresh pollen (or anther loculi or germinated grains) is dispersed on a microscope slide. For acetocarmine staining, they are suspended in a drop of acetocarmine (1 g refluxed in 45% acetic acid for 24 h and filtered), heated over a sprit flame, squashed and examined after 5-30 min. For DAPI staining, fresh or fixed pollen is stained on a slide in a drop of 0.005% DAPI (diamidinophenylindole), and the nuclei observed under a fluorescent microscope after 5-30 min (UV excitation, blue DAPI fluorescence). Permanent and semi-permanent slides can be made.

*Other Staining Methods*

Stanley and Linskens (1974) comprehensively review the other staining methods such as iodine-potassium iodide and tetrazolium red. While still useful for particular purposes, the FCR test has largely replaced the other tests because of its accuracy and ease of scoring.

**Germination Tests**

If pollen is able to germinate and produce a pollen tube, there is a high chance that it is viable and able to fertilize. Hence, for pollens where germination is possible *in vitro,* such tests are valuable.

In vivo *and semi-*vivo *tests*

These tests involve germinating the pollen on an unpollinated stigma. The stigma may be attached to the plant, or excised and planted in a 1% agar medium with 10-20% sucrose. The pollen tubes are stained in the style and observed within it or observed coming from the bottom of the style into the medium. The most widely used stain for pollen tubes



is decolorized aniline blue (DAB; 0.1% aniline blue stirred for 24 h in 0.1 M $K_3PO_4$; see Dumas and Knox 1983). The style is hydrolyzed in strong sodium hydroxide until softened, and mounted on a slide with gentle squashing, under a cover slip in DAB and sometimes 20% glycerin. After 30 min, epi-fluorescent light microscopy with UV or blue excitation will show the pollen tubes by white or yellow fluorescence.

Unlike other tests, hydration on the stigma occurs at the correct rate for the species, so incorrect pre-conditioning is less of a problem (see chapter on Pollen Hydration). Incompatibility reactions will normally be detected by microscopy, but even when they occur still give evidence that the pollen was potentially viable.

In vitro *germination tests*

*In vitro* germination of pollen is widely used for viability tests, under the generally correct assumption that pollen that germinates and produces a tube *in vitro* is likely to do so *in vivo*, and to fertilize the egg. Pollen grains from many species will germinate and grow on both solid and liquid artificial media. The most widely used media are modifications of the minimal medium of Brewbaker and Kwack (1964), consisting of 1 to 4 mM $H_3BO_3$ and 1 to 4 mM $Ca(NO_3)_2$ in 0.30 to 0.90 M (10-30% sucrose). Boron and calcium are often the only essential elements, although various workers add magnesium, phosphate buffers, flavenols, non-specific substances such as vegetable juice or yeast extract. More inert ingredients than sucrose (e.g. polyethylene glycol) have been used to prevent the grains bursting. The pollen is dispersed in droplets of liquid media on slides, which are often placed in humid atmospheres hanging upside down (hanging drops) to prevent anoxia, or in 0.5 ml of medium in a tube on a rotator. Alternatively, a semi-solid medium, with the addition of 0.5-1% agar can be used, and the pollen sprinkled on the surface. After 15 min (e.g. rye) to 12 h (e.g. lily), the pollen and tubes are examined by light microscopy. Fixation (in glutaraldehyde, acetic acid : ethanol, or ethanol) will preserve sample for later observation. Germination percentages, growth rate measurements and tube lengths are easily measured.

In general, binucleate pollen germinates readily in such media. Many trinucleate species also germinate, but others - wheat, for example - are recalcitrant and reliable media are not known.

Nevertheless, indications that flavenois such as quercetin can be added to media may give reasonable germination percentages in cultured tobacco pollen (Herbele-Bors et al. 1991) and wheat (Heslop-Harrison and Bardsley, unpublished). New media, e.g. for pistachio pollen germination (Golan-Goldhirsh et al. 1991), are regularly being found.

**Pollen Quality**

All the methods of testing discussed above depend on many variables and none is able to confirm that a particular sample of pollen is inviable and will not be able to fertilize any plant: they only give a likelihood estimate. Most are unsuitable for field use, and the variability between different samples for the same species, even although fertile, can be high. Nevertheless, cytological methods for testing pollen quality have proved valuable for research and in agriculture, and give unique information about plant fertility.

**References**

Brewbaker and Kwack (1964)
Cross JW, Ladyman JAR (1991) Chemical agents that inhibit pollen development: tools for research. Sex Plant Reprod 4:235-243.
Lu J, Mayer A, Pickersgill B (1990) Stigma morphology and pollination in *Arachis* L. (Leguminosea). Ann Bot 66: 73-82
Knox RB, Williams EG, Dumas C (1986). Pollen, pistil and reproductive function in crop plants. Plant Breeding Rev 4:9-79.
Dumas C, Knox RB (1983) Callose and determination of pistil viability and incompatibility. Theor Appl Genet 67: 1-10.
Golan-Goldhirsh A, Schmidhalter U, Mueller M, Oretli JJ (1991) Germination of *Pistacia vera* L. pollen in liquid medium. Sex Plant Reprod 4: 182-187.
Herbele-Bors et al. (1991) Como Meeting.
Heslop-Harrison J, Heslop-Harrison Y, Shivanna KR (1984) The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. Theor Appl Genet 67: 367-375.
Heslop-Harrison JS, Heslop-Harrison J, Heslop-Harrison Y, Reger BJ (1985) The distribution of calcium in the grass pollen tube. Proc Roy Soc Lond B, 225: 315-327.
Shivanna KR, Linskens HF, Cresti M (1991) Responses of tobacco pollen to high humidity and heat stress: viability and germinability in vitro and in vivo. Sex Plant Reprod 4: 104-109.
Stanley RG, Linskens HF (1974) Pollen: Biology, Biochemistry and Management. Springer Berlin.

# MEGASPOROGENESIS AND MEGAGAMETOGENESIS

M.T.M.Willemse
Dept. Plant cytology and morphology, Agricultural University,
Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

**Introduction.**

Megasporogenesis and megagametogenesis lead to the formation of the embryo sac. It includes the development of a highly differentiated plantlet in function of the sexual reproduction of angiosperms. After a meiotic division and cell isolation the development from the unicellular stage to the multicellular embryo sac represents the life cycle of the haploid plant. This life cycle from megaspore to megagametophyte with the megagamete takes place in and is strongly related to the mother-plant, the sporophyte.

**The life cycle of the megagametophyte.**

The functions of the megagametophyte are:
-to maintain contact with the mother plant to get nutrients
-to prepare the acceptation of the pollen tube
-to realize the double fertilization
-to prepare the start for the formation of the embryo and endosperm.
This implies that the megagametophyte is a very specialized organism!
The way to reach this functions occurs gradually during the life cycle in different stages in cooperation with the mother-plant. Variations on the different steps and sometimes phases leads to different types of development and embryo sacs. The most common is the Polygonum type.
During the life cycle the following stages with their main phases can be distinguished:
I. Preparative during megasporogenesis: the formation of one haploid isolated cell.
planning stage: with the formation of the archespore cell and induction of the meiosis.
transition stage: with the meiosis, transition to haploidy and to cell polarity.
selection stage: with the isolation and selection of the type of functional megaspore and uptake of storage for further development.
II. Differentiation during megagametogenesis: the formation a pollen tube

# The Angiosperm Stigma

J.S. Heslop-Harrison
Karyobiology Group
Department of Cell Biology
JI Centre for Plant Science
Norwich NR4 7UJ England

## Introduction

The angiosperm stigma is an efficient structure with both morphological and physiological adaptations that enable pollen capture, hydration and germination. The stigma surface may play a vital part in controlling interspecific hybridization and in regulating compatibility relationships within species. The structural and physiological features of the pollen capturing surfaces vary considerably between families and sometimes within families. This chapter aims to show the range of morphological variation in stigma types found throughout the angiosperms, to enable classification of stigmata into the major recognized groups, and to indicate the relationship between structure and function.

## The Flower

Floral structures are among the most important and variable characters used for plant identification. Indeed, many species can only be separated on floral characteristics. The variability arises because the flower is the structure with the highest complexity of any plant part - in number of sub-structures, tissue organization, and number of genes expressed. Despite the importance of the flower in taxonomy, little attention has been paid to one of its major sub-structures: the stigma. This contrasts with the pollen, where several textbooks exist with pictures and keys for plant identification based on pollen alone. This does, though, reflect current use of the character: pollen is often preserved in dry or wet conditions, potentially for thousands of years,

and is valuable for the identification of species composition of ancient ecosystems, and hence for discovery of the climate and other variables.

### The Stigma

The stigma is the receptive surface of the style that collects the pollen and enables its hydration and germination. The style connects the stigma to the ovule and includes the pollen tube transmitting tract. The style is involved in pollen tube guidance, nutrition and incompatibility responses. Angiosperm stigmata (or stigmas) are structurally very diverse and the surfaces adapted for pollen grain capture differ widely both in the morphology of the receptive cells and in the amounts of surface secretion. The first large scale systematic survey of stigmata from 250 families was carried out by Heslop-Harrison and Shivanna (1977), and a major reassessment of the taxonomic work on stigmata was presented by Heslop-Harrison (1981). These works, to which considerable reference is made here, described the major surface characteristics and morphologies of stigmata from 1000 species belonging to 900 genera, and developed a classification scheme for the various types of stigma (Table 1). The system divides species into two major groups: 1) those with stigmata that have a wet surface, bearing a fluid secretion, and 2) those where the stigma surface is dry and lacks any surface secretion; subdivisions separate species based on the presence of trichomes or papillae.

Table 2 gives the stigma types of species in a few major families that are of economic, scientific or horticultural interest. A much fuller listing of the characteristics of 250 families is given by Heslop-Harrison and Shivanna (1977).

### Study of the Stigma

The stigma is one of the most short-lived structures of a plant. Each stigma remains receptive to pollen for a few days at most, and in some species may be functional for only a few minutes after pollination. Many characteristics of the stigma can only be studied when it is in the receptive, mature state. Particularly in species with wet stigmata, substantial maturation may occur within the hours, or even minutes, immediately before the stigma becomes receptive. Surface fluids may be rapidly secreted, but in some species the stigma begins to autolyse



Table 1. A classification of Angiosperm stigma types based on the amount of secretion present during the receptive period and the morphology of the receptive surface. Abbreviations for each major type are given in the right hand column. (After Heslop-Harrison 1981).

| | |
|---|---|
| **Surface Dry** | |
| Receptive cells (trichomes) dispersed | |
| on a plumose stigma | D Pl |
| Receptive cells concentrated in | |
| ridges, zones or heads | |
| Surface non-papillate | D N |
| Surface distinctly papillate | |
| Papillae unicellular | D P U |
| Papillae multicellular | |
| Papillae uniseriate | D P M Us |
| Papillae multiseriate | D P M Ms |
| **Surface Wet** | |
| Receptive cells papillate; | |
| secretion moderate to slight, | |
| flooding interstices | W P |
| Receptive cells non-papillate; | |
| secretion usually copious | W N |

as soon as it becomes receptive. Therefore many characters can only be studied from fresh, mature stigmata, and the study of fixed or dried stigmata is inadequate. In agriculture, the effective pollination period (EPP) is an important character, and it is measured to find the time available for pollination of a plant variety. In some species, there may be little visible difference between receptive and non-receptive stigmata. For example, in avocado, the stigma accumulates callose as it senescences and becomes non-receptive (Sedgely 1977), although the difference between a receptive and non-receptive stigma is not visible without staining. Testing for stigma receptivity is difficult, although some methods are available (reviewed by Knox, Williams and Dumas 1986).

Observations of stigma surface morphology and secretions can often be made with a dissecting microscope or hand lens. However, the scanning electron microscope (SEM) is extremely valuable for observing and recording stigma morphology because of its high resolution, large depth of field and ability to examine untreated stigmata. The most informative SEM images are obtained using fresh, unfixed, and uncoated material; procedures including fixation, drying and coating are

usually unnecessary and generally add artifacts. A fresh stigma is excised and planted on a specimen stub in a viscous conductive solution - fish or animal derived glues with high salt concentrations, or graphite particle suspensions are usually suitable. The fresh specimen must be observed and recorded quickly once placed in the microscope chamber to avoid dehydration and charging.

For cytochemical and ultrastructural studies of the stigma, a wide range of techniques is used. Squashed preparations, often made as thick mounts, are valuable to show cellular morphology and differentiation in the stigma - unicellular or multicellular papillae can be observed easily, and the pollen tube pathways examined. Cytochemical studies (Pearse 1972; Gurr 1965) enable ions, proteins, lipids and carbohydrates to be stained specifically, and localized in preparations from fresh, unfixed material. For higher resolution studies, fresh, fixed or frozen stigmata can be studied by electron microscopy techniques (see Shivanna, Ciampolini and Cresti 1989; Heslop-Harrison 1990).

### The Dry Stigma (D)

A few genera, such as *Euphorbia,* have smooth dry domes of cells to which the pollen grains adhere and directly hydrate (DN), but most dry stigmata have trichomes (figure 1), unicellular (figure 2) or multicellular papillae. Pollen grains have a size appropriate to the papilla size and

Table 2. Representative families that include genera with the stigma characteristics shown in table 1. Some families have genera that are in more than one subdivision. See Heslop-Harrison and Shivanna (1977) for a classification of 900 genera.

| | |
|---|---|
| Gramineae | D Pl |
| Betulaceae | D N |
| Crucifereae | D P U |
| Ranunculaceae | D P U |
| Malvaceae | D P U |
| Liliaceae | D P U |
| Oleaceae | D P M Us |
| Geraniaceae | D P M Ms |
| Leguminosae | W P |
| Ericaceae | W P |
| Solanaceae | W P |
| Orchidaceae | W P |
| Liliaceae | W P |
| Ericaceae | W N |
| Umbelliferae | W N |



adhere on or between the papillae before hydration and germination (see Chapters on Pollen Hydration and Cresti, Pollen Germination, this volume).

#### *The Dry Plumose Stigma (DPl)*

Plumose stigmata are characteristic of the grasses (Gramineae; see figure 1). They are well adapted to collecting wind dispersed pollen, and there is a strong co-adaptation between the size of the pollen grains, the morphology of the stigma and the spacing and positioning of the trichomes. The area of the receptive surface varies from a few $mm^2$ in small species to over 1000 $mm^2$ in *Zea mays*, where the entire "silk" (stigma) can capture pollen. All grass stigmata have a dry surface, covered with a protein containing pellicle, and are adapted to enable rapid hydration and germination of the pollen grain: Heslop-Harrison (1987) has shown how pollen of rye may rehydrate and germinate within 2 min of attachment to the stigma.

#### *The Dry Papillate Stigma (DP)*

A few workers have examined the ultrastructure and cytochemistry of the stigma of species with dry, unicellular papillate (DPU) stigmata. An early study by Heslop-Harrison and Heslop-Harrison (1975) showed that the stigma papillae of *Crocus* had a loose, chambered cuticle overlaying a thick pectocellulosic wall. Later work (Heslop-Harrison 1987) showed the penetration of the pollen tube under the stigma cuticle in *Crocus.* Shivanna *et al.* (1989) described the thin, outer pellicle of the stigma of *Hypericum calycinum*, and showed that the cell wall of the papillar cells consisted of an outer, loosely woven fibrillar layer and more dense inner layer with compact fibrils. These wall characteristics enable pollen tubes to penetrate the cuticle, often near the base of the papillae, and enter the transmitting tract of the style, which may be either hollow or filled with cells. Dry papillate stigmata are generally (although not exclusively) associated with sporophytic self incompatibility, where the incompatibility factor carried on the surface of the pollen relates to the paternal genotype, and inhibition occurs at or near the stigma surface (see Thompson and de Nettancourt, this volume).

Some species with dry stigma surfaces have a copious secretion under a detached surface cuticle. The cuticle is normally ruptured by

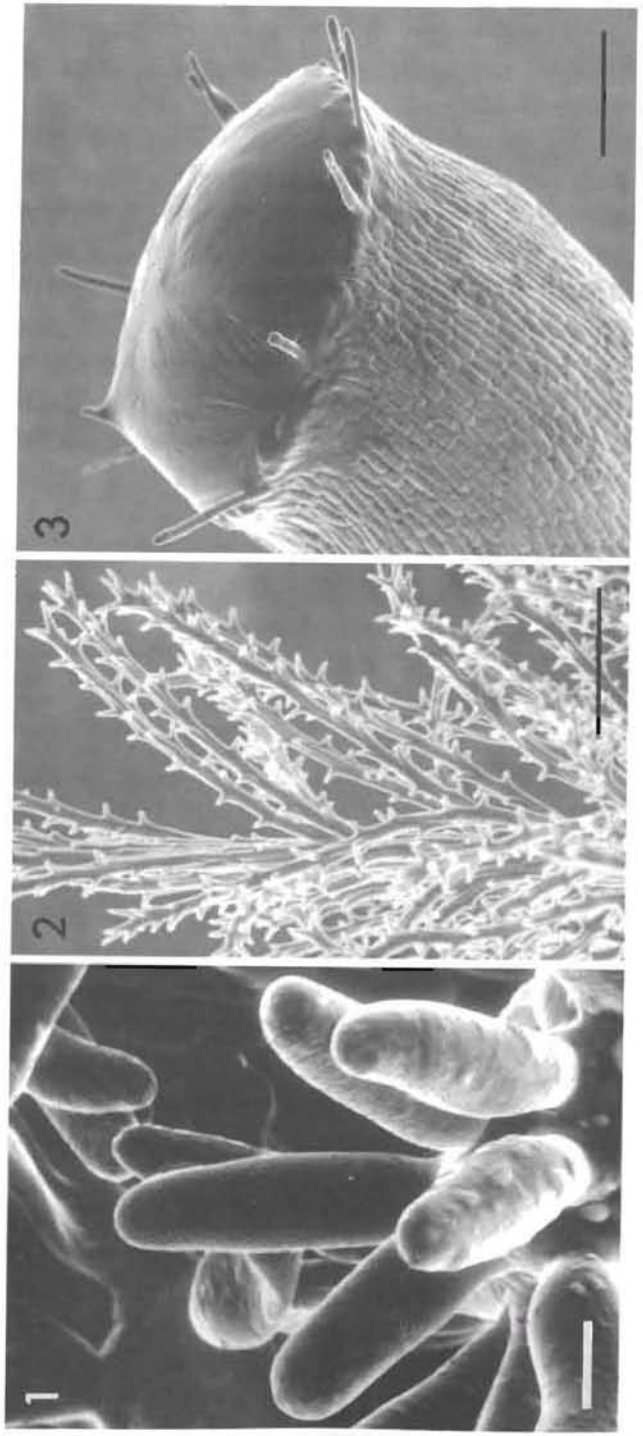
pollinating insects, which releases the secretion so that pollen hydration and germination can occur (Lord and Heslop-Harrison 1977). Under field conditions, different lines of *Vicia* show varying levels of autofertility, and analysis shows that the structure of the stigma varies between the lines. Those with thin cuticles over short papillae, that easily rupture, are much more autofertile than lines with thick cuticles and long papillae. Therefore, variations in stigma characters can be important for reproductive isolation of lines, and lead to differences in the field performance of crops.

**The Wet Stigma (W)**

Wet stigmata have a copious surface secretion. The secretion can be in a crater, or stigmatic cup, as in some Legumes (Owens 1990; figure 3), or on a dome, as in some lilies. Often, the secretion is far from homogeneous, consisting of both hydrophobic and hydrophilic components, and including lipids, proteins and carbohydrates. Konar and Linskens (1966a, b) showed a remarkable adaptation in the wet stigma of *Petunia*: it was stratified with a surface, lipid rich layer and a thin aqueous layer underneath. The pollen lands on the lipid layer and sinks through to the aqueous layer where hydration begins. This adaptation is also found in other Solanaceae, but not Rosaceae. Owens (1989) showed that there were dispersed lipid drops in the surface secretion of some legumes. The secretion also included sloughed off cells that arose from the loose, secretory tissue underneath the secretion; these secretory cells, and the large, intercellular spaces, could be seen clearly after critical point drying. Slater and Calder (1990) examined the ultrastructure of the detached cells in the stigmatic secretion of an orchid (*Dendrobium speciosum*) and found that the cells had all the characteristics of active secretory cells including extensive networks of endoplasmic reticulum, dictyosomes and vesicles.

Since materials carried on the surface of pollen grains are dispersed in the secretion, wet stigmata are associated only with gametophytic self-incompatibility systems, where the pollen genotype itself determines rejection or acceptance, and the rejection reaction usually occurs in the style.






Figures 1 to 3. Scanning electron micrographs of stigmata characteristic of the major groups in table 1. 1. *Crocus chrysanthus* (Iridaceae) with a dry, papillate, unicellular (DPU) stigma; bar 25 $\mu$m; (courtesy Dr Y. Heslop-Harrison, Aberystwyth). 2. A dry plumose (DPl) stigma from wheat (*Triticum aestivum*), characteristic of grasses. Most grass stigmata are bifurcated (not shown); bar 200 $\mu$m. 3. A wet papillate (WP) stigma from a *Senna nemophila* (Leguminoseae; courtesy Dr S.J. Owens, Kew); bar 100 $\mu$m.

### Stigma Turgidity

The stigma is usually strong and, particularly in the grasses with plumose stigmata, exerted from the flower. These characteristics are normally given by turgidity, and after pollination, the turgid stigmatic tissues often collapse and desiccate rapidly. While the surfaces of most plants are heavily protected from dehydration and pathogen attack, the stigma cannot be protected by a thick cuticle or wax since it must capture and hydrate pollen, and allow eventual penetration of the pollen tube into the intercellular spaces of the pollen tube transmitting tract. In the grasses, the stigma must remain turgid in any atmosphere and cannot be well protected when pollen is shed. Results from cytochemical and energy dispersive X-ray analysis of the elements present in the stigma show that potassium and chloride ions together account for 60% of the osmolality of stigma sap in *Pennisetum* (Heslop-Harrison 1990; Heslop-Harrison and Reger 1986). Thus these ions are very important for maintaining the turgidity of the stigmatic cells and, in this system as in the anther filament (Heslop-Harrison, Heslop-Harrison and Reger 1987), it is probable that regulation of potassium ion movement provides a rapid and sensitive method to control the cell osmoticum and to expand the stigma. A secondary effect of the high ion concentration makes the stigma electrically conductive, so that it does not charge strongly when examined under the electron beam of the SEM.

Some species in the Boraginaceae have an adaptation that may be related to water conservation. The papillar cells of the stigma have caps that touch to form a "roof". Pollen landing on the roof does not hydrate, while that which is forced between the papillae (perhaps by insects walking on the surface), hydrates and germinates rapidly, presumably in the moist atmosphere under the roof (Heslop-Harrison 1981). Hence the outer surface, forming the roof of each papillar cell, can have a thickened, impermeable wall, while the stalk, underneath the roof, can have a more permeable wall that enables hydration of pollen and penetration of the pollen tube.

### Pathogen Protection

The stigma also provides a potential point for fungal spores to germinate and penetrate the plant. In the cereals, ergot is a minor



fungal disease that can enter through the stigma, and a fungal mass (sclerotium or ergot) replaces the grain. Susceptibility of the plant seems to be related to the time the flower remains open, so plants with flowers that are pollinated and then close soon after the stigma becomes receptive are rarely infected. Male sterile plants of the normally cleistogamous barley, where the flowers remain open for a long time, are extremely susceptible to ergots - up to 76% of the heads may become infected, although fertile barley lines are rarely infected in the field (Agrios 1988). In some species, including *Zea mays,* the stigma abscises after pollination (Heslop-Harrison, Heslop-Harrison and Reger 1985), while in other species it becomes flaccid or dehydrates. The reasons for the generally high level of resistance to stigma infection are unknown, but it seems likely that there are several barriers to infection used by plants.

### Stigma Characteristics

Although characteristics of the stigma have rarely been considered in taxonomic and phylogenetic studies, clearly there are many regularities in stigma classification within genera and families. Although some families have stigmata of more than one type, evolutionary trends and intermediate states are often evident so stigma morphology can be used, like pollen morphology, for classification studies. Stigmata show substantial structural adaptations, and co-adaptations with pollen, to ensure efficient capture of pollen from the same species and rejection of alien, and sometimes self, pollen. Further structural features are important to enable pollen hydration, germination and pollen tube penetration, while minimizing water loss and pathogen invasion. Understanding the form and function of the stigma and its interaction with the pollen is a vital part of any study of sexual plant reproduction.

### References

Agrios GN (1988) Plant Pathology. 3rd ed Academic Press New York.

Gurr E (1965) The Rational Use of Dyes in Biology. Williams and Watkins Baltimore.

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. Int Rev Cytol 107: 1-78.

Heslop-Harrison J, Heslop-Harrison Y (1975) Fine structure of the stigmatic papilla of *Crocus*. Micron 6:45-52.

Heslop-Harrison J, Heslop-Harrison Y (1983) Pollen-stigma interaction in the Leguminosae: The organization of the stigma in *Trifolium pratense* L. Ann Bot 51:571-583.
Heslop-Harrison JS (1990) Energy dispersive X-ray analysis. pp 244-277 in Modern Methods in Plant Analysis New Series, Volume 11, Ed HF Linskens and JF Jackson Springer Berlin.
Heslop-Harrison JS, Heslop-Harrison Y, Reger BJ (1987) Anther-filament extension in *Lilium*: Potassium ion movement and some anatomical features. Ann Bot 59:505-515.
Heslop-Harrison JS, Reger BJ (1986) Chloride and potassium ions and turgidity in the grass stigma. J Plant Phys 124:55-60.
Heslop-Harrison Y (1976) Localisation of concanavilin A binding sites on the stigma surface of a grass species. Micron 7:33-36.
Heslop-Harrison Y (1981) Stigma characteristics and angiosperm taxonomy. Nordic J Bot 1:401-420.
Heslop-Harrison Y (1990) Stigma form and surface in relation to self-incompatibility in the Onagraceae. Nordic J Bot 10:1-19.
Heslop-Harrison Y, Heslop-Harrison J, Reger BJ (1985) The pollen-stigma interaction in the grasses. 7. Pollen-tube guidance and the regulation of tube number in *Zea mays* L. Acta Bot Neerl 34:193-211.
Heslop-Harrison Y, Shivanna KR (1977) The receptive surface of the angiosperm stigma. Ann Bot 41:1233-1258.
Knox RB, Williams EG, Dumas C (1986) Pollen, pistil and reproductive function in crop plants. Plant Breeding Reviews 4:9-79.
Konar RN, Linskens HF (1966a) The morphology and anatomy of the stigma of *Petunia hybrida*. Planta 71:356-371
Konar RN, Linskens HF (1966b) Physiology and biochemistry of the stigmatic fluid of *Petunia hybrida*. Planta 71:372-387
Lord EM, Heslop-Harrison Y (1984) Pollen-stigma interaction in the Leguminosae stigma organization and the breeding system in *Vicia faba L*. Ann Bot 54:827-836.
Owens SJ (1989) Stigma, style, pollen and the pollen-stigma interaction in *Caesalpinioideae*. Advances in Legume Biology Monogr Syst Bot Missouri Bot Gard 29:113-126.
Owens SJ (1990) The morphology of the wet, non-papillate (WN) stigma form in the tribe *Caesalpinieae (Caesalpinioideae: Leguminosae)*. Bot J Linn Soc 104:293-302.
Owens SJ, Lewis GP (1989) Taxonomic and functional implications of stigma morphology in species of *Cassia, Chamaecrista* and *Senna (Leguminosae: Caesalpinioideae)*. Plant Syst Evol 163:93-105.
Pearse AGE (1972) Histochemistry - Theoretical and Applied. 2 vols, 3rd ed Churchill, London.
Sedgely M (1977) Reduced pollen tube growth and the presence of callose in the pistil of the male floral stage of the avocado. Sci Hort 7:27-36.
Shivanna KR, Ciampolini F, Cresti M (1989) The structure and cytochemistry of the pistil of *Hypericum calycinum*: The stigma. Ann Bot 63:613-620.
Slater AT, Calder DM (1990) Fine structure of the wet, detached stigma of the orchid *Dendrobium speciosum* SM. Sex Plant Reprod 3:61-69.

# Pollen Capture, Adhesion and Hydration

J.S. Heslop-Harrison
Karyobiology Group
Department of Cell Biology
JI Centre for Plant Science
Norwich NR4 7UJ England

## Introduction

Pollen capture by the stigma initiates a complex sequence of events that can lead to the hydration and germination of the pollen grain, and eventually to penetration of the pollen tube and fertilization. This chapter will review aspects of the physiology and physics of events related to pollen capture, hydration and adhesion, but not concepts related to the physical capture of pollen by the stigma. Work on the aerobiology of pollen capture in species pollinated by wind is reviewed by Niklas (1987), and in water pollinated species by Ducker and Knox (1976). Many ecological works discuss aspects pollen transfer animal vectors.

Pollen dehydration occurs during the final stages of maturation within the anther, and presumably continues up to and after dehiscence. Since the mature pollen is almost inactive metabolically (Hoekstra and Bruinsma 1980), hydration levels may fluctuate with changes in atmospheric conditions. Once the pollen lands on a stigma, rehydration can occur very rapidly, leading to regaining of active metabolism: in rye (*Secale cereale*), hydration starts within a few seconds of a pollen grain landing on the stigma, and in *Brassica*, germination often occurs within ten minutes of pollen capture. Study of fast events, particularly when they involve an interaction between the pollen and stigma, is difficult. Some aspects of hydration and the early events preceeding germination can be studied *in vitro*, by placing pollen in liquid media or on semi-solid media, but active interactions must be studied on the stigma.

**Dehydrated and Hydrated Pollen Structure**

Dehisced pollen grains have widely varying hydration status between species, ranging from 6% water content in *Populus* to 60% in *Zea mays*. Because all routinely used biological fixatives are aqueous, and hence change hydration status, it is difficult to study dehydrated pollen by cytochemical methods or electron microscopy. However, an understanding of the events of dehydration - and rehydration - is important for fundamental research and for practical applications, since the understanding will help to overcome barriers to hybridization or limitations of fertility caused by problems associated with rehydration, and find new methods for the long term preservation of germplasm or breeding materials.

The thickened and often sculptured pollen wall, the exine, their high lipid content and the refractile nature of most pollen grains, prevent useful imaging inside the grain using light microscopy. Conventional fixation of hydrated and unhydrated grains in glutaraldehyde, for electron microscopy, indicated that there are substantial differences in internal structure between grains plunged into fixative and those hydrated before fixation (Heslop-Harrison 1979). More recently, vapour phase and cryo-fixation methods have been used for electron microscopy of dehydrated and hydrating pollen without the possibility of rehydration, and physical imaging methods have examined membranes within grains.

Anhydrous vapour fixation of unhydrated, partially and fully hydrated grains of *Brassica* was used by Elleman and Dickinson (1986) to show the change in pollen ultrastructure during hydration. Grains were placed in a closed jar containing osmium tetroxide vapour, that pre-fixed the grains without changing hydration, before aqueous fixation for electron microscopy. A previously undetected, membrane-like coating superficial layer (CSL) was found around the pollen grains, which is important since this structure is the first to contact the stigmatic pellicle. However, striking changes in cytoplasmic organization were particularly noteworthy providing remarkable evidence that the membranes changed from a micellar form to a hydrated, continuous form in a few minutes after hydration.



Tirawi, Polito and Webster (1990) used freeze substitution to look at unhydrated (7% moisture content) and hydrated viable pollen of pear in the electron microscope. Grains were quench frozen in liquid propane in an unhydrated state or after 2 min in a germination medium, before freeze-substitution with 1% osmium tetroxide in acetone, and embedding in resin. The data showed that the ultrastructure of the hydrated pollen grains was extremely different from that of dry pollen. Dry pollen showed a shrunken cell morphology: there were infoldings of the wall and a crowding of the organelles. However, there were vesicles associated with the extracytoplasmic, as well as the cytoplasmic, surface of the plasma membrane, and many multilamellate membranes and dense osmiophilic bodies in the cytoplasm, that were presumably associated with the dehydrated status but not simple shrinking. In the grains that were hydrated for two minutes, the cell wall infoldings, intine wall corrugations, osmiophilic bodies and the multilamellate membranes disappeared, and the grains showed an ultrastructure generally typical of plant cells. The authors suggest that the complex, tightly packed, multilamellate membrane conserves membrane material, which become surplus as the grain dehydrates, in a form that is ready rapidly to reform the membrane system of the cell upon rehydration.

Crowe, Hoekstra and Crowe (1989) were able to examine the state of membranes within bulk samples of pollen by Fourier transform infrared spectroscopy. Their results demonstrated a significant change in the structure of the membrane between the hydrated and dehydrated state. Using pollen from *Typha latifolia*, they showed that the membrane phospholipids in the dry pollen were in a gel phase (dry bilayer). If a grain is then placed into water (or a medium), the gel phase phospholipids change to a liquid crystalline phase (hydrated bilayer), and the grain contents leak through the membranes during the phase transition. The cytoplasmic leakage causes pollen death. However, when a grain is rehydrated over water vapour, or heated slightly, the gel phase lipids undergo the transition to the liquid crystalline phase without leakage. When the grain is subsequently placed into water, there is no leakage of the grain contents through the hydrated bilayer in the liquid crystalline state, so the grain remains viable and can germinate. Thus it is vital for the dry pollen to hydrate under conditions

that allow the membrane phase change to occur without leakage of the grain contents for viability to be maintained.

The model of pollen hydration proposed by Heslop-Harrison (1979) suggested that the plasma membrane is porous and ineffective at the time of dispersal so it does not provide any osmotic barrier. Membrane integrity is restored by rehydration on the stigma after pollination. The data discussed above strongly support this model. The hydration of the pollen grain either on a stigma or on an artificial medium is effected by the passage of water through the germination apertures of the grain, where the intine is more accessible. The apertures themselves often show refined adaptations for the regulation of water loss and uptake (Heslop-Harrison, Heslop-Harrison and Heslop-Harrison 1986). If rehydration is too rapid, considerable imbibitional leakage of the cytoplasm occurs, leading to death of the pollen. In the dehydrated grains of many species, including hazel and rye, the apertures are sealed either by 'lids' of by infolding of the lip-like markings of germinations slits, which is completed by the coalescences of the surface lipids (Heslop-Harrison et al 1986; Heslop-Harrison 1987). Contact with a moist surface leads to dispersal of the lipid, exposing the underlying intine that then allows hydration. As the intine hydrates, the slits gape more and water intake is further facilitated.

### Stigma Interactions

The work above discusses the changes in pollen upon hydration *in vitro*, and similar changes occur when the grain hydrates on a stigma. As discussed by Heslop-Harrison (1987), successful pollination requires a high level of co-adaptation between the pollen and the stigma and style. At the time of pollen capture and during germination, the interactions involve the female sporophyte and the male gametophyte, with surface, paternally derived, materials carried by the pollen grain. Breakdown of precise co-adjustment at any point will act as a fertility barrier. Because of the randomness associated with most pollination systems, stigmata commonly capture pollen from foreign species, and this is generally rejected. There can be crude maladjustments - size of pollen and stigma papillae, or water tensions in the stigma and pollen can prevent initial grain hydration, or cause osmotic bursting of the pollen - although



specific interactions are also important (Heslop-Harrison and Heslop-Harrison 1975).

### Attachment to the Stigma

In species with the dry type of stigma, the capture of pollen depends upon its attachment to the surface of the papillae, but the attachment is apparently more complex than simple adhesion. Maize pollen transferred to the stigma is held, but easily dislodged. During the first 3-5 minutes, the adhesion increases in strength until germination of the grain. Ferrari et al (1985) demonstrated the several stages of binding in compatible combinations in *Brassica*. The first "force", begining seconds after pollen contact with the stigma, was dissociated in methanol or sodium hydroxide, but not water. A second binding force, which was dissociated only in sodium hydroxide, occurred later, after germination, a third, sodium hydroxide stable, force bound the germinating grain to the stigma. They discuss their results in terms of van der Waals forces and lipid polymerization, although such forces would tend not to give the specificity shown by the work of Salker, Elleman and Dickinson (1988).

### Hydration on the Stigma and Recognition Reactions

In many angiosperms, pollen from the same or genetically identical individuals is rejected - the plant is self-incompatibile. In all sporophytic and some gametophytic systems (see chapters by de Nettancourt and Thompson), the stigma surface is the site of self-incompatibility responses, and it is now clear that the compatibility recognition events can occur even before the pollen is fully hydrated, at least in *Brassica*. Indeed, the initiation of hydration of the grain by passage of water from the stigma, and the transfer of pollen wall proteins from the pollen exine and intine to the stigma surface occur simultaneously (Heslop-Harrison and Heslop-Harrison 1985). However, the pollen-stigma interactions are complex, and environmentally modified, so present descriptions are incomplete.

In compatible *Brassica* pollinations, hydration of the grain on the stigma is often complete within five minutes following capture of the grain, and in this period, emissions from the pollen wall sites are transferred to the stigma surface (Heslop-Harrison 1975). In compatible

combinations, germination begins in 3-10 minutes and the tube penetrates the cuticle in 10-30 min. In an incompatible reaction, the rejection responses are first evident in 5-10 min.

Zuberi and Dickinson (1985) assayed the hydration of *Brassica* pollen grains by measuring their length to width ratio, which is related to the grain volume and hence hydration state. The pollen grains could take up water from a humid atmosphere as well as the stigma, but long periods (3 h) on a slide at high humidity lead to the pollen becoming inviable. On the stigma, dehydrated pollen immediately started hydrating, and, in compatible pollinations, the process continued until germination had taken place. However, the rate of hydration depended on the S genotypes of both the pollen and stigma, as well as the compatibility between the two. In humid (low vapour pressure deficit) conditions, pollen tube organization and penetration were adversely affected. Although the pollen tube usually germinated, its growth tended to become disorganized after emergence from the grain and it was not able to penetrate the papillar surface. Thus, the grain can obtain water both from the stigma and from the atmosphere, and some early parts of the incompatibility reactions (leading to rejection of the pollen by the stigma) were overcome by hydration from the atmosphere. Thus they could conclude that hydration plays a key role in the operation of the sporophytic self-incompatibility system in *Brassica*.

### Control of Hydration

The regulated passage of water from stigma to pollen is an essential prerequisite for successful pollen tube development. Heslop-Harrison (1979) advanced a hypothesis that slow hydration by water from the atmosphere and the stigma enables the developing pollen grain to initiate the process of cytoplasmic reorganization that is crucial for normal germination and penetration of the stigma, and then gave a theoretical consideration of the water potentials of the different stages of hydration. Elleman and Dickinson (1986) presented data to confirm the model: the dry pollen first extracts water from the stigma by matric potential, and then, after formation of an intact plasm membrane, by a system based on turgor pressure differentials. Exudation of water from the grain can occur at an intermediate stage. The water flow is regulated by a balance between the hydraulic pull exerted by the pollen grain and the resistance to flow provided by the stigma. The structures involved in this process, reviewed by Sarker, Elleman and Dickinson (1988), are complex: the pollen is bounded by a lipidic coating invested by a membrane-like coating superficial layer (CSL; see above), while the stigma surface consists of an enzyme rich superficial pellicle investing an irregular cuticular layer. The papilla boundary is well adapted for the regulated passage of water to the pollen through its cellulosic wall with microchannels, and osmotic changes may open cracks in the cuticle to enable more rapid water flow, and later penetration of the pollen tube.



The events within the pollen grain following hydration are the subjects of other chapters in this volume (see Pierson, Cresti). These events are activated and occur rapidly after hydration, but clearly stigma attachment and the precise regulation of hydration is an important event in initiation of the pollen stigma interaction in many species.

### References


Crowe JH, Hoekstra FA, Crowe LM (1989) Membrane phase transitions are responsible for imbibitional damage in dry pollen. Proc Natl Acad Sci USA 86:520-523.

Ducker SC, Knox RB (1976) Submarine pollination in seagrasses. Nature 263:705-706.

Ferrari TE, Best V, More TA, Comstock P, Muhammad A, Wallace DH (1985). Intercellular adhesions in the pollen-stigma system: Pollen capture, grain binding, and tube attachments. Am J Bot 72:1466-1474.

Gaget M, Saied C, Dumas C, Knox RB (1984). Pollen-pistil interactions in interspecific crosses of *Populus* (sections Aigeiros and Leuce): Pollen adhesion, hydration and callose responses. J Cell Sci 72:173-184.

Heslop-Harrison J (1975) Male gametophyte selection and the pollen-stigma interaction Gamete competition in Plant and Animals. DL Mulcahy. North Holland, Amsterdam.

Heslop-Harrison J (1979) An interpretation of the hydrodynamics of pollen. Am J Bot 66:737-743.

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. Int Rev Cytol 107:1-78.

Heslop-Harrison J, Heslop-Harrison Y (1985) Surfaces and secretions in the pollen-stigma interaction: A brief review. J Cell Sci Suppl 2:287-300.

Heslop-Harrison J, Heslop-Harrison Y 1975. Ann Bot 39:163-165

Heslop-Harrison Y, Heslop-Harrison JS, Heslop-Harrison J (1986) Germination of *Corylus avellana* L. (Hazel) pollen: Hydration and function of the oncus. Acta Bot Neerl 35:265-284.

Hoekstra FA, Bruinsma J (1980) Control of respiration of binucleate and trinucleate pollen under humid conditions. Physiol Plant 48:71-77.

Knox RB, Williams EG, Dumas C (1986). Pollen, pistil and reproductive function in crop plants. Plant Breeding Rev 4:9-79.

Niklas KJ (1987) Pollen capture and wind-induced movement of compact and diffuse grass panicles: implications for pollination efficiency. Am J Bot 74:74-89.

Sarker RH, Elleman CJ, Dickinson HG (1988) Control of pollen hydration in *Brassica* requires continued protein synthesis, and glycolsylation is necessary for intraspecific incompatibility. Proc Natl Acad Sci USA 85:4340-4344.

Stead AD, Roberts IN, Dickinson HG (1979) Pollen-pistil interactions in *Brassica oleracea*: Events prior to pollen germination. Planta 146:211-216.

Tiwari SC, Polito VS (1988) Spatial and temporal organization of actin during hydration, activation and germination of the pollen in *Pyrus communis* L.: a population study. Protoplasma 147:5-15.

Tiwari SC, Polito VS, Webster BD (1990) In dry pear (*Pyrus communis* L.) pollen, membranes assume a tightly packed multilamellate aspect that disappears rapidly upon hydration. Protoplasma 153:157-168.

Zuberi MI, Dickinson HG (1985). Pollen-stigma interaction in *Brassica* III. Hydration of the pollen grains. J Cell Sci 76:321-336.