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Pollen Grains and Tubes

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MORPHOLOGY AND ANATOMY | Pollen Grains and Tubes

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Abstract

A knowledge of pollen functions and its development in the genus *Rosa* is often of the greatest importance to the rose breeders and scientists when planning a hybridization program and research. The following paragraphs, therefore, brings together all the available scientific information from pollen formation to fertilization and its management.

Keywords

pollen morphology, pollen development, pollen tube, pollen germination, pollen fertilization, reproductive barriers, pollen viability, *in vitro* germination, cryopreservation, unreduced gametes, pollen size

Introduction

Fundamental biological functions of pollen in the sexual reproduction of plants are now well known. Firstly, the haploid microgametophyte is the widespread vector of recombinant characters after male meiosis. Secondly, the pollen grain, deposited on the female stigma, germinates by forming a pollen tube from an aperture of pollen wall, in order to lead to double fertilization. Many processes occur during this stage such as pollen germination, pollen tube growth, movement of hormonal molecules and specific gene activation. In the genus *Rosa*, as in many other genera, pollen, as a reproductive unit, plays a major role in various aspects of the life cycle of the plant. Its formation is one of the highest energy-consuming processes of the plant and its release is influenced by genetic and environmental factors. As a male sexual component, pollen contributes theoretically half of the characters of the paternal parent (except for the *Caninae* section, roses with asymmetric male/female meiosis) (*see*GENETICS | Meiosis; GENETICS | Inheritance in the Dogrose). For wild roses, pollen contributes significantly to spatial and temporal gene flows. For the 'manmade' roses, high pollen quantity, loading from a limited number of male parents, is manipulated and managed principally in order to give directional genotypes with valuable characteristics. A review of current knowledge of rose pollen in relation to these different aspects is made here.

Pollen Morphology

At the macroscopic level, rose pollen, like the pollen of many rosaceous species, looks like a very fine yellow colour dust, constituted by numerous pollen grains with an elongated or more and less elliptical shape. This fine powder is released when the pollen sacs open at the stage of anther dehiscence (**Figure 1**). Under the light microscope, fresh rose pollen has an elongated form (**Figure 2**) with a constant ratio of length to width equal to 2.The diameter of rose pollen grains may vary from 5 µm to 70 µm, according to species, hybrids, ploidy level and environmental conditions (Pécrix et *al.* 2011 *Journal of Experimental Botany*). In particular, diploid (2*n*) pollen has a diameter 1.2-1.3 times larger than that of haploid (*n*) pollen (Jacob and Pierret 2000 *Acta Horticulturae* 508; 289-292). In hybrid tea roses, pollen morphology is characterized as small (<30 µm), shrunken, irregular (abnormal) or large (>30 µm),

elliptical and crossed by furrows (normal). In cut roses the percentage of normal pollen and larger diameter is related to high pollen fertility (Pipino *et al.* 2010 *Acta Horticulturae* 870: 143-146; Pipino *et al.* 2011 *Euphytica* 178: 202-214).

Figure 1. Fresh pollen released from isolated stamens in a plastic Petri dish, 24 h after collection from fresh flowers. Pollen ‘dust’ is visible on the surface of the dish.

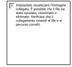
Figure 2. Fresh pollen of a diploid rose (*Rosa rugosa* var. *rubra*) observed by an optical microscopy without any staining. The elongated form of the pollen grain and some apertures are visible.

Rose pollen structure is similar to that of other rosaceous species. The visible part of the pollen grain is the exine which is the outer layer of a living pollen grain. This layer is mainly constituted of a polymer called sporopollenin, and small quantities of polysaccharides. Sporopollenin, formed from the polymerization of carotenoids, is chemically very stable and is resistant to almost all kinds of environmental damage. In roses, the exine is equipped with three apertures (three pores: pollen is thus described as ‘tricolpate’ or ‘triporate’) from which the pollen tube emerges upon germination in the pollination process, leading to fertilization. When observed under the scanning electron microscope, the exine of rose pollen grains shows some structural and anatomical patterns that are correlated with phylogenetic background of the species that produced them. In rose species, the mean exine thickness is about 1.10 µm. One of the thinnest species is *R. spinosissima* (about 0.70 µm), while one of the thickest is *R. pendulina* (about 1.99 µm). The exine is divided into two sublayers: the outermost sexine and the unsculptured underlying nexine. In roses species or cultivars, the sexine patterns differ mainly in their aperture membranes, length and density of ridges and furrows and in the density and spatial patterns of small perforations. These features either did not occur or are very scarce in *R. persica*, *R. banksiae*, and in diverse species from the section Pimpinellifoliae. These structures give the exine a striated or reticulated appearance with several levels of combinations of one or both characteristics. In the majority of species, the exine is striate, although in *R. multibracteata* and *R. multiflora* the exine is

striate-psilate and blurred striate (Wrońska-Pilarek and Jagodziński 2011 *Plant Systematics and Evolution* 295: 55-72). The inner layer, called the intine, is mainly composed of cellulose and is very similar in construction to ordinary plant cell walls (**Figure 3**).

Figure 3. Schematic representation of the morphology of a rose pollen grain.

Pollen Formation

Pollen is the result of two sequential stages. The first stage is the microsporogenesis (*see*GENETICS | Meiosis) followed by the second stage, microgametogenesis, the process of transformation of a microspore in a pollen grain. Microspore development consists of an expansion of its volume, which is commonly associated with the formation of a vacuole. This is also the stage of sporopollenin polymerization and the formation of the final pollen wall structure as previously described. During this stage, the microspore nucleus moves from a central position to an eccentric one, close to the wall. In this position the first pollen mitosis (pollen mitosis I) occurs and gives two unequal cells. The larger one is the vegetative cell which contains a large and diffuse haploid nucleus. The other cell is the generative cell with a small and condensed nucleus. This cell is contained within the cytoplasm of the vegetative cell. In roses, the generative nuclei in mature pollen contains a 2*n* DNA amount whereas the vegetative cell contains only a 1*n* DNA amount. This means that, even if the second pollen mitosis (pollen mitosis II) occurs in the pollen tube (**Figure 4**), DNA replication is performed into the generative cell, inside the pollen grain. This pollinic mitosis occurs without any cytokinesis (cell division).

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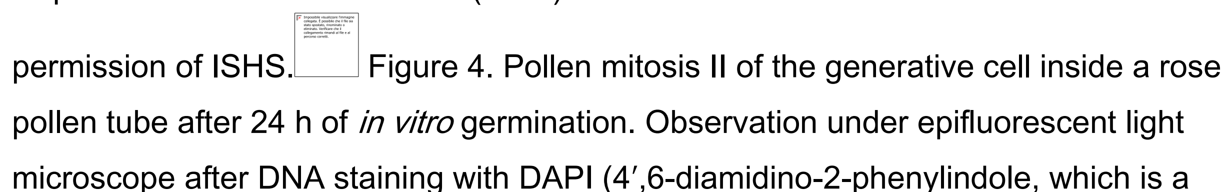


Figure 4. Pollen mitosis II of the generative cell inside a rose pollen tube after 24 h of *in vitro* germination. Observation under epifluorescent light microscope after DNA staining with DAPI (4′,6-diamidino-2-phenylindole, which is a

specific-DNA fluorescent stain). Two sperm nuclei (G) are clearly visible whereas the vegetative nucleus (V) is in proximal position in the pollen tube's extremity.

Pollen Release

In rose flowers, stamens (when they are present) are composed of the filament, which is the stalk connected with the floral receptacle, and the anthers, where the pollen is present, which are attached to the filament by a sterile parenchymatous tissue called connective. Each anther is constituted with two symmetric pollen bags (thecae) fixed to the connective. At flower maturity, pollen is generally released by conjunction of external and internal factors but the critical stage of dehiscence is basically the initial stage of pollen spreading. For example, on *R. rugosa*, pollen is generally released from the stamens when the flower bud is still closed. In contrast, on rose cultivars with a high level of duplication, pollen may be released later after flower bud opening. External factors such as ambient air humidity, temperature and solar radiation can also influence rose pollen release. Very diverse amount of pollen per flower can be found within the genus *Rosa*. *R. rugosa* produces between 19.1 and 45.9 mg of pollen per flower, while much lower abundance is produced in *R. canina* (3.3 mg per flower) and in *R. multiflora* (2.0 mg per flower) (Wronska-Pilarek *et al.* 2015 *Palynology* 39(1): 56-75; Zuraw et *al.* 2015 *Acta Agrobotanica* 68(3): 267-278). Natural vectors of pollen are insects (*see*INSECTS AND OTHER ANIMALS | Overview of Insects). Rose breeders currently use some techniques to accelerate pollen release in order to manage the hybridization process. Anthers can be collected after removal of petals on defined flower bud stages. Then, to induce the complete pollen release, anthers are left for 24 h in open Petri dishes at a mean temperature of 25°C and hygrometry equal to 60%. When pollen is produced in great quantity, anther dehiscence can also occur after 12 h in a growth chamber at 30°C (*see*BREEDING | Methods of Cross Breeding).

Pollen Germination and Fertilization

Pollen tube growth in roses displays similarities to that in other Rosaceae and, by extension, other genera. When a rose pollen grain lands on a stigma, it adsorbs water

and a rehydration process occurs. Various signals, especially those of pollen–stigma interaction, induce an intense metabolic activity in the pollen grain which start to grow and forms a pollen tube. The pollen tube invades the pistil, growing between the walls of the stigmatic cells, then travelling through an extracellular matrix within the transmitting tissue of the style. The pollen tube finally arrives at the ovary, where it is attracted to an ovule that contains an egg cell. This phase of pollen development, called the progamic phase, corresponds to the phase occurring between pollination and fertilization. In roses, the pollen tubes reach the ovules between 12 and 24 h after pollination. Fertilization is believed to occur within this interval of time. The growth of the pollen tube has been studied at the molecular level on model plants such as *Arabidopsis thaliana*, and several genes involved in pollen hydration, germination and tube guidance have been identified (Maruyama *et al.* 2014 *Plant & Cell Physiology* doi: 10.1093/pcp/pcu018; Lin *et al.* 2014 *The Plant Cell* 26: 602-618). The role of volatile molecules on pollination events are also described on the model plant *Petunia* x *hybrida* (Muhlemann *et al.* 2014 *Plant, Cell & Environment* 37(8): 1936-1949). The primary pollination event is accompanied by an increase in ethylene evolution in the stigma and style within hours after pollination and well before pollen germination. While, among plant hormones, auxin is proposed to serve as the primary pollination signal by the direct transfer of pollen-borne auxin to the stigma (Kovaleva and Zakharova 2003 *Sexual Plant Reproduction* 16: 191-196). Pollen tubes are themselves considered to be a model for cellular growth of plant cells.

Some external factors are well known to influence rose pollen germination strongly. Important variation in stigma pH can be found between genotypes. This factor, known to be involved in the pollen germination response, depends on the genotype, and plays a role in the fertilization response and consequently in seed set. Ionic elements like boron and calcium which are involved in the metabolism on pollen tube growth, and at different concentrations can stimulate or inhibit it. Naturally occurring polyamines such as spermine also stimulate pollen tube germination and elongation when they are sprayed on the stigma. *In vitro* techniques for rose pollen germination are positively applied by using germination media containing boric acid (H_3BO_3), sucrose, putrescine,

and agar at pH equal to 5.0. The fresh pollen is dusted directly onto sterile Petri dishes with the medium. Temperatures between 23-30°C and a relative humidity about 60-65% are the best conditions for *in vitro* pollen germination and pollen tube elongation. In hybrid tea roses the mean percentage of pollen germination is positively correlated with the percentage of normal pollen.

Self-Incompatibility

In roses, as in other Rosaceae, reproductive barriers like mechanisms of self-incompatibility (SI, inability of pollen to fertilize the ovule of the same plant) occur. It has been determined that the type of self-incompatibility found in *R. rugosa* is a gametophytic mechanism. This means that all compatible or incompatible pollen starts to germinate, but growth of incompatible pollen tubes stops in the style while compatible tubes go on to fertilize the egg in the ovule. The existence of SI has been confirmed also in *R. multiflora* by self pollination and cross-pollination by using many clones of this species (Debener *et al.* 2010 *Acta Horticulturae* 870: 183-190). The SI response is typically manifested within the upper third of the style where incompatible pollen tubes exhibit reduced rates of elongation, loss of membrane integrity, disrupted organelles, and wall thickening, all of which can lead to swelling and bursting of the tube tip. This dramatic cessation of pollen tube elongation is effected by the S-RNase (*S*-locus ribonuclease), an abundant and highly polymorphic pistil-specific glycoprotein encoded by the *S* locus and secreted into the extracellular matrix that lines the path of pollen tube growth (Sijacic *et al.* 2004 *Letters to Nature* 429: 302-305). By extension, this gametophytic self-incompatibility (GSI) probably occurs in the majority of self-incompatible roses. The GSI system, which is genetically controlled by a single-locus, multiallelic family of genes, has also been dissected at the molecular level in other Rosaceae and also in other model plants. Self-incompatibility system widely exists in the genus *Rosa*, especially in diploid species. In tetraploid and hexaploid species, the break-down of self-incompatibility is found and it is thought that such self-compatibility arises through competitive interaction of *S*-alleles in diploid and triploid pollen. To assess self-incompatibility or compatibility in

wide crosses, pollen germination and pollen tube growth can be observed by fluorescence microscopy with classical protocols developed on other species such as the aniline blue staining procedure (Table 1). In roses, fertilization is difficult to observed with these techniques, due to the presence of natural fluorescence of stylar tissues that mask fluorescence in the pollen tube wall. (Figure 5).

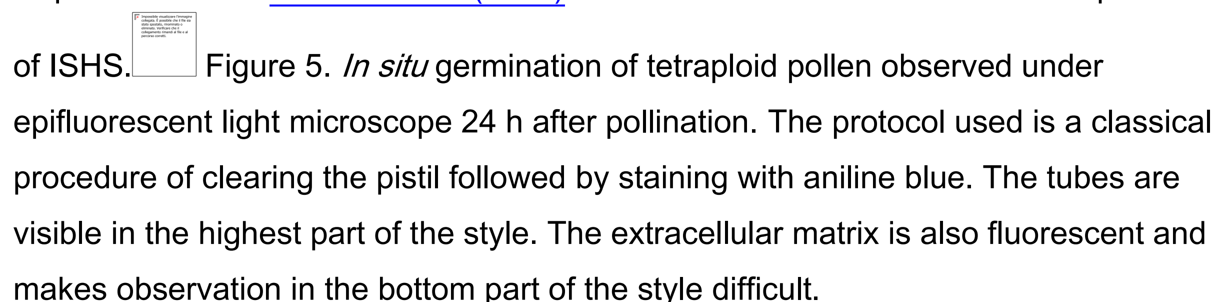
Reproduced from Jacob Y *et al*. (2001)*Acta Horticulturae* 547: 383–385 with permission of ISHS. Figure 5. *In situ* germination of tetraploid pollen observed under epifluorescent light microscope 24 h after pollination. The protocol used is a classical procedure of clearing the pistil followed by staining with aniline blue. The tubes are visible in the highest part of the style. The extracellular matrix is also fluorescent and makes observation in the bottom part of the style difficult.

Table 1.

Cytological staining procedures

Procedure	Materials		Method
Acetocarmine staining	Glacial acetic acid	45 ml	Heat the 45% (w/v) acetic acid solution to boiling. Add the 0.5 g carmine and continue heating for several minutes with stirring. Filter and cool. Pollen is put on slides, stained with a few drops of acetocarmine solution and cover-slipped. Under light microscope, the pollen cytoplasm of viable pollen grains appears in red.
	Water	55 ml	
	Carmine	0.5 gm	
Alexander staining	Ethanol 95% (w/v)	10 ml	Pollen is put on slides, stained with a few drops of Alexander solution and cover-slipped. After 15–20 min, the pollen is observed under light microscope, its wall being coloured in green and the cytoplasm of viable pollen grains in red–purple.
	Malachite green [1% w/v in 95% v/v ethanol]	1 ml	

Procedure	Materials		Method
	Fuchsin acid (1% w/v in water)	5 ml	
	Orange G (1% w/v in water)	0.5 ml	
	Phenol	5 g	
	Chloral hydrate	5 g	
	Glacial acetic acid	2 ml	
	Glycerol	25 ml	
	Distilled water	50 ml	
Aniline blue staining	Aniline blue (1% w/v in water)	1 part	Several hours after pollination, flowers are collected and finely sliced from stigma to the floral peduncle. They are placed in the mix and boiled for 10 min. Before observation, they were placed in the following mix to improve fluorescence: samples are put on slides, mounted with a drop of aniline blue (1%) in K_3PO_4 0.1 M, cover-slipped and observed under fluorescence microscope equipped with subsequent filters (340–380 nm excitation filter).
	Tween 20	2 parts	
	K_3PO_4 (0.2 mol l^{-1})	7 parts	
	Sodium hydroxide (1 mol l^{-1})	1 part	

Pollen Management: Storage and Viability

Rose pollen has largely been studied in order to optimize its management, mainly for the benefit of rose breeders. To facilitate crosses in breeding programmes, efficient protocols to assess rose pollen vitality have been developed, and protocols for long-term conservation of pollen have been studied. One of the characteristic of rose pollen

that can be underlined is its great variability in terms of differences of pollen characteristics between different genotypes. The large genetic diversity in this genus, the various interspecific combination abilities, the range of ploidy levels and the intense effort to produce horticultural roses are each a part of the reasons that can explain this variability. Moreover, rose breeders have attempted to enrich the variability of the gene pool of cultivated roses by introducing germplasm of new diploid wild species. Haploidization programme of cultivated roses is developed to the ploidy level of cultivated roses from tetraploid to diploid (Mokadem *et al.* 2002 *Euphytica* 125: 169-177). Dihaploids of tetraploid rose cultivars can be obtained also by irradiated pollen. A wild rose will generally produce a homogeneous and functional pollen population with null or a very low proportion of dead, empty or abnormal pollen grains. In contrast, many horticultural hybrids from various origins produce pollen populations with a low to very high percentage of inefficient pollen grains (unable to fertilize). Various male meiotic or postmeiotic disturbances can generally explain the presence of dead, sterile or abnormal pollen. Microscopic observations under visible or ultraviolet light, after specific staining, can be performed to detect easily a part of these abnormalities. For example, staining with aceto-carmine stain, or Alexander stain is currently used for this purpose (Table 1) In particular, after hydration, small abnormal pollen maintains more or less the original shrunken and irregular shape. In tetraploid hybrid tea roses, dry abnormal pollen shows a diameter lower than 30 µm with no furrows on the surface typical of aborted pollen. Among these pollen characteristics, the presence of unreduced male gametes (pollen with chromosome number higher than half of the chromosome number of the father plant) can easily be detected due to the relation between pollen size and ploidy level of the pollen (Figures 6 and 7). The ploidy level of rose pollen can also be estimated with flow cytometry techniques, which measure the amount of DNA in individual pollen grains. The bead beating technique was used to prepare suspensions of plant nuclei to estimate ploidy of *R. canina* pollen (Roberts 2007 *Cytometry Part A* 71A: 1039-1044).

Figure 6. Pollen of a diploid species (*Rosa rugosa* var. *rubra*) stained with acetocarmine (only viable pollen is stained in red) and observed under light microscope. Pollen size is very regular. Viability level is very high (close to 100%).

Figure 7. Pollen of a tetraploid variety (JV6 cv. Saint Trop) stained with acetocarmine and observed under light microscope. Pollen size is larger than the pollen of diploid roses and some 'jumbo pollen' attached to unreduced pollen occurs. Viability level is highly variable depending on the particular cultivar.

The occurrence of unreduced pollen can significantly disturb heredity studies. Indeed, a mix of normal and unreduced pollen is able to induce progenies with different ploidy levels. However, presence of unreduced pollen must be turned as an advantage for the breeder, particularly when adjustment at a superior ploidy level is found. For example, a diploid rose, able to produce unreduced pollen and crossed with a tetraploid plant, can give theoretically tetraploid plants in the progeny. More generally, unreduced pollen grains can be a way to sexually polyploidize roses. This mechanism is believed to have played a significant role in the origin of rose polyploidy (*see*GENETICS | Karyology). Rose breeders may overcome geographic distances and differences in flowering time by storing pollen of the desired male parents until pollination of the female plants can be performed. Breeding programmes are often focused on the quality of rose pollen which is genotype dependent and it is affected by the conditions used for its conservation. In fact, rose pollen is functional when it is freshly collected and keeps a certain viability during the following days of pollen release. When the pollen is stored at ambient temperature during several weeks its efficiency decreases rapidly. While, rose pollen tube length of pollen stored for two or 52 weeks at -80°C is comparable to fresh pollen and longer than that of pollen stored at -20°C or 4°C (Giovannini et al. 2015 *Acta Horticulturae* 1064: 63-66). Recent studies on hybrid tea roses shows that the viable level of fresh pollen varies among cultivars and also the pollen preservation at -20°C and -80°C is cultivar dependent (Macovei et al. 2016 *Notulae Botanicae Agrobotanici Cluj-Napoca* 44(1): 6-10). Different freezing conditions have shown that normal freezing can increase the period of usability of pollen up to 2 to 3 months. For long-term storage,

cryopreservation of rose pollen is an efficient method. Pollen can apparently retain a high level of viability after 1 year in liquid nitrogen. Pollen degradation during storage conditions could be due to dehydration, which results in loss of pollen colloidal properties. In addition, as reported in seeds, reactive oxygen species (ROS) and reactive nitrogen species (RNS) over-accumulation can inhibit pollen germination. Various investigations have been carried out to estimate the pollen viability and male fertility of rose cultivars. Viability and fertility can be estimated by *in vitro* pollen germination tests. These tests, which are done in a simple medium containing sucrose, boric acid and deionized water, allow the observation of pollen tube growth. However, these tests often overestimate pollen ability to grow and to fertilize.

Conclusion and Prospects

The pollen of roses shows morphological, anatomical and genetic characteristics which find applications in several domains. Pollen studies contribute to a better knowledge of phylogenetic relationships in the genus *Rosa* and constitute an aid for the complex botanical and horticultural classifications of roses. In various species, recent developments in the knowledge of pollen biology have been achieved particularly at molecular level (i.e. fine mechanisms of pollen stigma interaction, pollen tube guidance, ploidy level, unreduced gametes, etc…). These developments applied to rose pollen should allow progress towards a better management of hybridizations and consequently a means to optimize rose breeding programmes. At both professional and amateur levels, rose breeding activities should be stimulated by transfer of simple methods (i.e. microscopy control of pollen quality; evaluation of pollen size; *in vitro* germination and viability tests) to ensure the best possible efficacy of rose pollen manipulation. Finally, in terms of pollen storage, modern protocols (i.e. cryopreservation and deep-freezing preservation) could significantly contribute to long-term management of rose germplasm.

See also

BIOTECHNOLOGIES FOR BREEDING | Manipulation of Ploidy Level; BREEDING | Methods of Cross Breeding; GENETICS | Karyology; GENETICS | Meiosis; GENETICS | Inheritance in the Dogrose; INSECTS AND OTHER ANIMALS | Overview of Insects; INSECTS AND OTHER ANIMALS | Pollination.

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Change History

January 2017. M Caser added new information about the state of art in Introduction and Conclusion and Prospects sections and updated Pollen Morphology, Pollen Release, Pollen Germination and Fertilization, Self-Incompatibility and Further Reading sections. The Sections Pollen Management and Pollen Storage and Viability were merged in

Pollen Management: Storage and Viability and updated. New relevant citations were added in the text.