

Pollen–pistil interaction is unique to flowering plants. This is one of the critical postpollination phases that determine reproductive success. Following conspecific compatible pollination, pollen grains germinate on the receptive stigma and the resulting pollen tubes grow through the tissues of the stigma and style and enter the ovules where they deliver the sperm cells for fertilization. Pollen–pistil interaction involves a series of sequential events from pollination until the pollen tube enters the embryo sac. A break at any level in these sequential events results in the failure of fertilization. There is a continuous dialogue between the pollen grain and later the pollen tube and the tissues of the pistil. Successful completion of pollen–pistil interaction is a prerequisite for fertilization and subsequent fruit and seed development.

Pollen–pistil interaction was taken for granted by the early investigators, partly because of lack of suitable techniques to study pollen germination and pollen tube growth in the pistil and partly because of their preoccupation with the development of the male and female gametes and the embryo and endosperm. However, during the last three to four decades, there has been an increasing realization of the importance of pollen–pistil interaction in reproductive biology of flowering plants, and considerable amount of information is now available (Shivanna 2003; Higashiyama and Hamamura 2008; Chapman and Goring 2010; Kawashima and Berger 2011).

8.1 Evolutionary Significance of the Pistil

The main function of the pistil is to screen pollen grains for compatibility and quality during pollen–pistil interaction. Only the right type (compatible) of pollen grains are facilitated to complete pollen–pistil interaction, and the wrong (interspecific pollen as well as self-pollen in self-incompatible species, *see* Chap. 9 for details) types are inhibited during their germination or pollen tube growth (*see* Shivanna and Johri 1985; Shivanna 2003; Lord 2003; Cheung et al. 2010).

Generally, the number of pollen grains deposited on the stigma is many times more than the number of ovules that are available in the ovary for fertilization. This creates intense competition even amongst the compatible pollen grains during pollen–pistil interaction. Pollen grains which germinate early and the pollen tubes which grow faster in the style enter the ovules earlier and effect fertilization; pollen grains which germinate later and pollen tubes which are slow growing are eliminated in this competition (Mulcahy 1979, 1984; Mulcahy and Mulcahy 1983; Armbruster et al. 2002; Shivanna 2003; Lankinen and Madjidian 2011). There is no scope for such a competition amongst the male gametes in lower groups of plants; any compatible male gamete that comes in contact with the female gamete, irrespective of its quality, fuses with the female gamete. There are evidences to indicate that pollen

competition in flowering plants increases the fitness of the progeny; the progeny resulting from rapidly growing pollen tubes are superior over the others that result from slow-growing pollen tubes (Mulcahy et al. 1988; Ottaviano et al. 1980; Davis et al. 1987; Snow and Timothy 1991). Evolutionary success of flowering plants over the other groups of plants has been suggested to be the result of the evolution of the most efficient outbreeding system (based on self-incompatibility) and intense pollen competition (Whitehouse 1950; Mulcahy 1979; Armbruster et al. 2002), both of which are the attributes of the evolution of the pistil.

8.2 Pollen Germination and Pollen Tube Growth

The stigma is the recipient of the pollen. The major events that occur on the stigma surface are pollen adhesion, pollen hydration and pollen tube entry into the stigma. Each of these events is a prerequisite for the subsequent event (Lord 2003; Shivanna 2003; Chapman and Goring 2010; Cheung et al. 2010). Pollen adhesion is not critical in species with wet type of stigma but is critical in those with dry type of stigma. The adhesive carbohydrates, lipids and proteins present on the pollen surface and/or the stigma surface play a crucial role in pollen adhesion and hydration. In members of Brassicaceae, the presence of pollen coat substances seems to be essential for pollen adhesion and hydration. Pollen coat substances are released onto the pellicle surface and form a meniscus in the interface which enables not only pollen adhesion but also facilitates pollen hydration by signalling movement of water from the stigma to the pollen. Many mutants with defects in pollen coat substances as well as pollen grains, from which pollen coat substances have been removed, fail to hydrate (Doughty et al. 1993; Dickinson 1995; see Zinkl et al. 1999; Shivanna 2003). In *Arabidopsis* also, pollen grains which have defects in lipid biosynthesis cannot hydrate on the stigma surface. Flavonoids which form a part of the pollenkit have been shown to play an important role in

pollen germination and pollen tube growth (Preuss et al. 1993). Flavonoid-deficient mutants of maize and *Petunia* produce flavonoid-deficient pollen which cannot function on the stigma of mutants. However, exogenous supply of nanomolar concentration of kaempferol, a flavonoid aglycone, either to pollen or to the stigma restored germination of mutant pollen (Mo et al. 1992).

Many earlier studies have shown that the stigma provides boron and calcium which are required for pollen germination in several species. In *Gladiolus* washing of the stigma with a detergent removed the ability of the stigma to support pollen germination, and in *Raphanus* enzymatic digestion of pellicle reduced pollen germination and totally inhibited pollen tube entry into the stigma (see Shivanna 2003). Thus, the pellicle seems to have many components, some of which are involved in germination and others in the entry of pollen tubes.

Pollen tube entry into the stigma is another critical step. In wet stigma types, the cuticle of the stigma surface is disrupted during secretion of the exudates, and thus there is no physical barrier for the pollen tube entry into the intercellular matrix of the stigmatic tissue. In dry type of the stigma, the cuticle forms a physical barrier for pollen tube entry. The pollen tube has to erode the cuticle by activation of cutinases. Cell wall hydrolases are present in the pollen coat. There are evidences to indicate the involvement of pollen xylanase in pollen tube penetration. Suen and Huang (2007) produced transgenic lines (*xyl-less*) containing little or no xylanase in the pollen coat of maize pollen. *xyl-less* pollen germinated as efficiently as wild-type pollen in vitro but less so on the silk (stigma) of corn. Once germinated, the *xyl-less* and wild-type pollen tubes elongated at comparable rates. However, pollen tubes of *xyl-less* pollen did not penetrate into the silk as efficiently as the tubes of wild-type pollen, and this lower penetration efficiency could be overcome by the addition of xylanase to the silk. These results clearly indicate that maize pollen coat xylanase facilitates pollen tube penetration into silk via enzymatic xylan hydrolysis.

In species with solid style, pollen tubes grow through the extracellular matrix (ECM) of the

stigma and eventually enter the ECM of the style. In hollow-styled species such as *Lilium*, in which the stylar canal is filled with secretion products from the canal cells, pollen tubes grow down on the surface of the canal cells. In species such as *Gladiolus* and *Crocus*, in which the mucilaginous substances accumulate between the cuticle and the canal cells, pollen tubes enter the cuticle and grow through the mucilage. The amount of nutrients present in the pollen is limited and not sufficient to support pollen tube growth in the style until it reaches the ovary. Obviously, the stylar tissue has to provide nutrients to the growing pollen tubes. Earlier studies using labelled sugars in the pistil have demonstrated that sugars are taken up by the growing pollen tubes from the style (see Shivanna 2003). Although the details of chemical interaction between pollen tubes and stylar components are not clearly understood, the ECM obviously plays a critical role in these interactions. Some studies have indicated that transmitting tissue-specific (TTS) proteins present in the ECM play an important role in pollen tube growth (see Lord 2001; Cheung et al. 2010).

8.3 Pollen Tube Guidance

Pollen tubes follow a predetermined path in the pistil from the stigma to the ovary. Traditionally it was thought that the pollen tubes are guided from the stigma to the ovary by a gradient of chemotropic substance present in the pistil. For some time it was believed that calcium is the chemotropic agent (see Shivanna 2003). However, most of the later evidences did not support the concept of the presence of a chemotropic substance in the pistil. Many investigators suggested that the architecture of the transmitting tract in the stigma and style with elongated, cylindrical cells interconnected at their ends to form vertical files, surrounded by the ECM, provides a continuous pathway of least mechanical resistance for growing pollen tubes. When once the pollen tubes enter the transmitting track, they follow this path unidirectionally from the stigma to the ovary (see Heslop-Harrison and Heslop-Harrison 1986). Although many experimental studies have

confirmed lack of chemotropic gradient in the style, some evidences have indicated that TTS proteins may provide a chemotropic gradient in the style in *Nicotiana* (Cheung et al. 1995).

Irrespective of the presence or absence of a chemotropic gradient in the style, there has to be some factor(s) at the level of the stigma to guide the pollen tubes into the stigma from its surface and in the ovary where in they have to change the direction of growth from the placenta to the ovule by about 90°. There has been significant progress in understanding these factors (Lord 2003; Shivanna 2003). In *Nicotiana*, characterized by the wet stigma, there are convincing evidences to show that the lipidic stigmatic exudate establishes a water gradient which ensures directional growth of pollen tubes into the stigma. The composition of the stigmatic exudates is such that it is permeable enough for pollen hydration to occur but not so permeable for water supply to become non-directional (Wolters-Arts et al. 1998). However, in *Brassica* and *Arabidopsis* with dry type of stigma, the lipids present on the pollen grain surface are involved in pollen tube guidance. They are released soon after pollination and come in contact with the stigma surface to form a contact zone, which provides a medium to establish directional water gradient for pollen hydration, germination and pollen tube guidance into the stigma (Wang et al. 1993; see Shivanna 2003).

Several studies, using in vitro assay, in *Arabidopsis* and *Torenia* have provided strong evidences to show that synergids of the embryo sac are involved in directing the pollen tubes from the placenta into the embryo sac (Hulskamp et al. 1995; Higashiyama et al. 2001; Punwani and Drews 2008). The filiform apparatus of the synergid seems to play an important role in guiding the pollen tube. A number of synergid-expressed genes and their proteins which are secreted into filiform apparatus have been identified (see Polanivelu and Preuss 2006; Okuda et al. 2009; Li et al. 2011; Kawashima and Berger 2011). These proteins have been suggested to play a role in the formation and/or function of filiform apparatus and possibly in pollen tube guidance.

Pollen–pistil interaction has now become an active area of research. Advance techniques of molecular biology and genetics are being used to unravel the mysteries of pollen–pistil interaction. In the coming years, significant advances are likely to be made in our understanding of pollen–pistil interaction.

8.4 Double Fertilization

Following the entry of pollen tubes into one of the synergids, pollen tube discharges the two male gametes into the synergid. One of the sperm cells approaches the egg and fuses with it to give rise to the zygote. The other sperm cell moves towards the central cell and eventually fuses with the fused polar nuclei (secondary nucleus) to give rise to the primary endosperm nucleus (Russell 1992; Russell and Dumas 1992). Although cytoskeletal elements have been implicated in the movement of the sperm cells, recent studies have indicated that the cytoplasmic flow of the pollen tube content generated by pollen tube discharge is enough to move sperm cells into the intercellular region between the egg cell and the central cell (*see* Kawashima and Berger 2011). The zygote gives rise to the embryo and the primary endosperm nucleus to the endosperm. Fertilized ovules develop into seeds and the ovary into fruit.

8.5 Protocols

8.5.1 Aniline Blue Fluorescence Method to Study Pollen Germination and Pollen Tube Growth in the Pistil

Details of pollen germination and pollen tube growth in the pistil are required to understand a number of aspects of reproductive ecology such as pollen viability, stigma receptivity and breeding system (Fig. 8.1). Most of the earlier studies depended on staining pollinated pistils in acetocarmine or aniline blue in lactophenol, making a squash preparation and observing them under a light microscope. Although pollen germination could be easily observed in such preparations, pollen tube growth could not be seen clearly in many species with thicker stigma and style. Some investigators used clearing of the pistil or dissecting the transmitting tissue of the pistil before staining. Lack of suitable techniques was one of the major limitations for studies on pollen germination and pollen tube growth by early investigators. Following the standardization of the technique of aniline blue fluorescence (Linskens and Esser 1957; Martin 1959; Shivanna and Rangaswamy 1992), it has become a standard method for studying various aspects of pollen

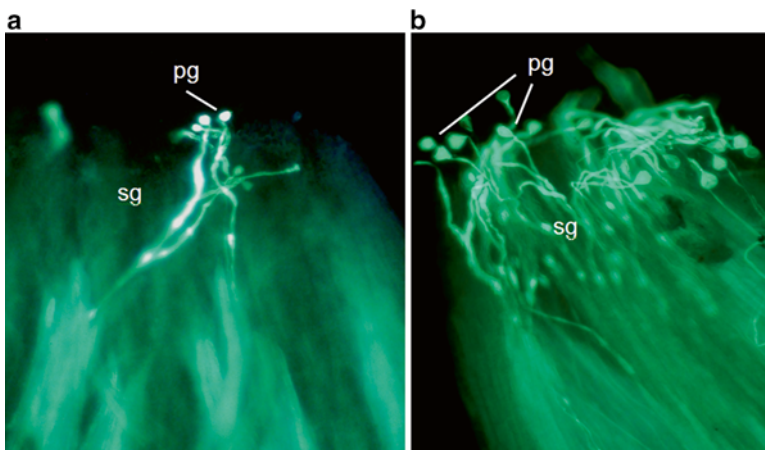


Fig. 8.1 Stigma receptivity in *Syzygium* sp. (a) Fluorescence micrograph of a cleared stigma stained with decolourized aniline blue, pollinated soon after anthesis. Stigma (sg) is not fully receptive and has supported ger-

mination of a few pollen grains (pg). (b) Stigma similar to a but pollinated 24 h after anthesis. Stigma is fully receptive and supported profuse pollen germination

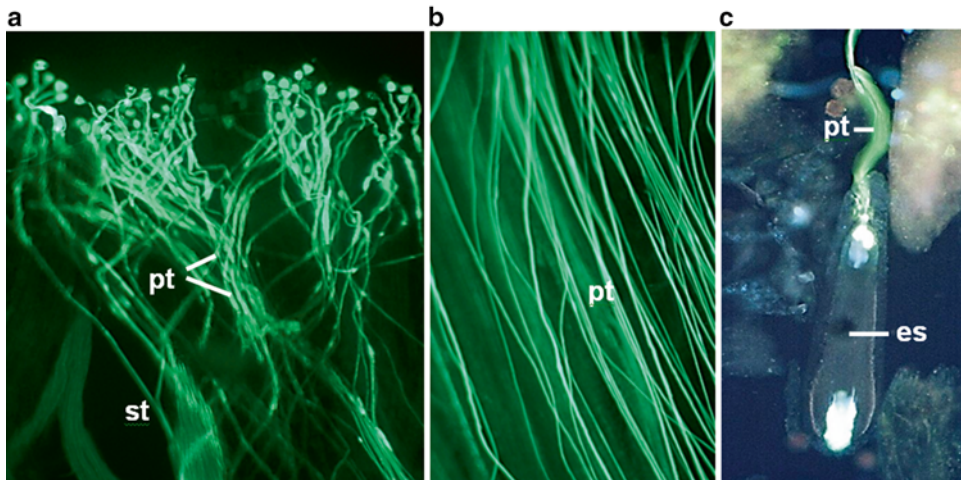


Fig. 8.2 Fluorescence micrographs of cleared aniline blue-stained preparations. (a, b) The stigma (a) and style (b) of pollinated pistil of *Syzygium* sp. to show profuse germination of pollen grains (*pg*) on the stigma and

growth of pollen tubes (*pt*) in the style (*st*). (c) An ovule of *Tribulus terrestris* to show pollen tube (*pt*) entry into the embryo sac (*es*)

germination and pollen tube growth and considerable literature has accumulated on this aspect. Aniline blue is a callose-specific fluorochrome. As pollen tubes invariably contain callose along the wall and also as callose plugs across older region of pollen tubes, aniline blue-stained preparations show bright fluorescence under fluorescence microscope with UV illumination (Fig. 8.2).

8.5.1.1 Special Requirements

Decolorized aniline blue stain (see Appendix A.1).

Sodium hydroxide 4–8 N.

Fixative: Many investigators have used FAA or acetic alcohol or LA (see Appendix A.1).

Fluorescence microscope.

8.5.1.2 Procedure

1. Fix the pollinated pistils in a fixative for about 24 h and store them in 70 % ethanol until use.
2. Transfer the fixed pistils to 4–8 N NaOH for clearing depending on the thickness of the pistil. For delicate pistils, 4 N NaOH is sufficient, while the robust pistils require 8 N NaOH. The period of clearing also varies based on the thickness of the pistils. At laboratory temperature, clearing is generally done

overnight. The period of clearing may be reduced by increasing the temperature to about 60 °C. After clearing, the pistils become rather soft and need careful subsequent handling. When LA is used as fixative, clearing may be done using 4 N NaOH or 4 N KOH. Lactic acid simultaneously fixes and also clears the tissue to some extent. KOH has been found to be useful in removing the phenolics.

3. Transfer cleared pistils to water taken in a Petri plate and rinse them carefully at least twice.
4. Mount the pistils in 1:1 mixture of aniline blue and 10 % glycerin.
5. Apply gentle pressure on the cover glass to spread the tissue.
6. Observe the preparations under a fluorescence microscope with UV filter combination (refer to Appendix A.8). Sieve plates of the phloem and often xylem elements also show some fluorescence and confuse the identity of the pollen tubes. The sieve plates and xylem elements can be identified by observing the same field under bright light; the thickenings of the xylem elements and the position of sieve plates along the xylem tissue help in their identification. The preparations can be stored for a few weeks in a refrigerator; however, care should be taken to prevent them from drying.

8.5.1.3 Modifications

1. Some investigators leave cleared and rinsed pistils in the stain for a few hours or overnight for staining and then mount in glycerin.
2. Resin-embedded sections or wax-embedded serial sections of the pistils or ovary along with ovules may be stained directly with aniline blue to localize pollen tubes in the transmitting tissue of the pistil or in the micropyle of the ovules.

8.5.2 Temporal Details of Pollen Germination and Pollen Tube Growth

In several studies, it becomes necessary to determine the rate of pollen germination and pollen tube growth. This is particularly important in self-incompatible species. In some self-incompatible species, pollen tubes may reach the base of the style in both self- and cross-pollinated pistils, but the growth of self-pollen tubes is much slower when compared with cross-pollen tubes; in such species even when pollinations are carried out with mixed pollen (self and cross), most of the seeds are sired by cross-pollen. Such studies are also needed in interspecific crosses. In this protocol, pollinated pistils are fixed at various time intervals after pollination and the extent of pollen tube growth is determined.

8.5.2.1 Special Requirements

Tags and pollination bags

Other requirements as in Protocol 8.5.1.

8.5.2.2 Procedure

1. Tag sufficient number of flower buds before anthesis and anther dehiscence. Divide them into several sets and label each set as i, ii and so on. The number of sets depends on the frequency of time intervals needed; to some extent this depends on the time taken for the pollen tubes to reach the base of the style. The number of flower buds for each set should be at least six.
2. Emasculate all the flower buds and bag them.
3. On the day of anthesis or when the stigma becomes receptive, open the bags and carry

out pollination with required pollen sample in all the sets of flowers and rebag them. Note down the time of pollination.

4. Excise pollinated pistils at preselected intervals (starting with set i) and fix them in a fixative. Generally, interval of 2–4 h is sufficient in short-styled flowers; in long-styled flowers such as tobacco, 6–12 h may be suitable.
5. Process each set of pistils separately to study pollen germination and pollen tube growth through aniline blue fluorescence method following Protocol 8.5.1.
6. Observe each pistil from each set and record the extent of pollen germination and the length of pollen tube growth in the pistil. One of the standard methods to measure the length of pollen tube growth in the pistil is to put a dot (on the mounted cover glass) with a marker at the region on the pistil up to which the pollen tubes have traversed, and then measure the length (mm/cm) from the tip of the stigma up to the dot.
7. After recording the extent of pollen germination and pollen tube growth in all the pistils of all the sets, calculate, for each set, average length up to which pollen tubes have grown in the pistil. Calculate the rate of pollen tube growth using the data from all the sets. Growth rate in the pistil is generally expressed as $\mu\text{m/h}$.

8.5.2.3 Modifications

1. Pollen tube growth can also be studied by pollinating excised flowers/pistils maintained in a beaker or a Petri plate (*see* Protocol 9.6.1).
2. To compare the rate of pollen tube growth in self- and cross-pollinated pistils, divide emasculated flower buds into two sets. Carry out self-pollinations on flowers of one set and cross-pollinations on the other set. Divide each set into subsets for fixing at regular intervals. And follow later steps as described above.
3. For studying the speed of pollen germination, fix pollinated pistils at 15 min intervals and score percent pollen germination/number of pollen grains germinated on the stigma (*see* Protocol 6.3.5 for details).

8.5.3 Semi-Vivo Technique to Study Pollen–Pistil Interaction

This method is a combination of in vivo and in vitro methods. The method can be used for studies on pollen vigour, temporal details of pollen tube growth and the division of the generative cell in 2-celled pollen systems. The flowers are pollinated in situ on the plant or after implanting them in a beaker containing water or agar medium set in Petri plates (see Protocol 9.6.1). Pollen germination and pollen tube growth are allowed to proceed in the pistil up to a desired length and the style is cut in front of the growing pollen tubes and implanted in a liquid or agar medium for the pollen tubes to emerge in vitro into the nutrient medium through the cut end of the style. This method has been used in several species with robust pistils such as *Oenothera*, *Nicotiana* and *Lilium*. Some of the aspects studied using semi-vivo technique are self-incompatibility (Niimi 1982), effect of ovary on pollen tube growth (Mulcahy and Mulcahy 1985), isolation of sperm cells in two-celled pollen species (Shivanna et al. 1988), pollen vigour (Shivanna et al. 1991) and the factors responsible to attract pollen tubes into the micropylar region of the ovules (Higashiyama et al. 2001; Higashiyama and Hamamura 2008).

This exercise needs some preliminary studies to standardize suitable medium for pollen tube emergence in vitro and to get some idea about the rate of pollen tube growth in the pistil.

8.5.3.1 Special Requirements

A suitable nutrient medium that permits satisfactory pollen tube growth. As a general guideline, a medium standardized for pollen germination would be suitable. For several species, we have used the medium containing sucrose (10 %), boric acid (100 mg/ml) and calcium nitrate (300 mg/ml) – either semisolid (0.8 % bacto agar) or liquid. Addition of polyethylene glycol may enhance pollen tube growth (see Appendix A.4).

Petri plates or beakers of suitable size.

8.5.3.2 Procedure

1. Emasculate mature flower buds before anther dehiscence and bag them.
2. On the day of anthesis or when the stigma becomes receptive, pollinate the emasculated flower buds with fresh pollen grains and rebag them.

The number of sets of pistils to be used for pollination depends on the objective of the study. For example, if the study is designed to compare the rate of pollen tube growth in self- and cross-pollinated pistils, two sets of pistils (one for self- and one for cross-pollination) have to be pollinated. Each set should have at least 6 pistils.

Also, the period for which pollinated flowers have to be left in situ depends on the length of the style through which pollen tubes have to grow in vivo and the nature of the study. For several studies, the growth of pollen tubes for 0.5–1.0 cm in the style is suitable; this usually takes 8–24 h depending on the species. However, the required period has to be checked.

3. Prepare suitable liquid/solidified agar medium for pollen tube growth and have the agar medium set in Petri plates or beakers depending on the size of the explant to be used.
4. At the end of the selected period, excise the pollinated pistils and bring them to the laboratory.
5. Cut the style transversely with a sharp blade in the region just ahead of the growing pollen tubes in the style.

This has to be done carefully without smudging the cut end. In hollow-styled species, hold the stylar part to be cut immersed in the liquid germination medium (used for pollen tube emergence) and then only cut the style. Otherwise air bubbles may get trapped in the stylar canal which may affect pollen tube emergence.

6. Immediately after cutting the style, implant the cut end in the liquid medium/agarified medium set in Petri plate/small beaker. If a liquid medium is used, keep the cut end of the

pistil dipped in the medium through small holes made in aluminium foil or parafilm used as lid for the beaker/Petri plate.

- Maintain the implanted pistils for 12–24 h at 22 ± 2 °C or at laboratory temperature.

Incubation time may vary depending on the species, the length of the style retained and the objective of the study.

- Observe periodically the cut ends of the style until pollen tubes emerge. Note the time of pollen tube emergence (from stylar implantation) and allow pollen tubes to grow 1–2 mm into the medium.
- After sufficient growth of the pollen tubes, gently pull out the style along with the emerged pollen tubes; place the cut end in a drop of the liquid medium; count the number of pollen tubes emerged and measure the length of pollen tubes. If the number of pollen tubes is too much, measure the length of randomly selected pollen tubes. If necessary, emerged pollen tubes along with a very short length of the cut end of the style may be cut from the remaining part of the style and used for mounting.

By staining pollen tubes with a DNA fluorochrome, such as DAPI, generative cell/male gametes and vegetative nucleus may be observed in the pollen tubes.

8.5.3.3 Modifications

- Instead of carrying out pollinations using flowers maintained on plants, pollinations can be carried out on flowers/inflorescences/flowering branches excised and maintained with their cut end dipped in water in the laboratory (for details *see* Protocol 9.6.1).
- Pollen vigour can be effectively assessed through semi-vivo method by timing the emergence of the pollen tubes after pollinating each set of the pistils with different pollen samples.
- This method can also be used to study stigma receptivity. Pollinate flowers of different ages/stages, allow a fixed time for pollen germination in all of them, cut the style a few mm below the stigma and implant in a medium. Pollen tubes emerge earliest in the pistils with

receptive stigma. In others pollen tubes may not emerge or emerge much later than that in receptive stage.

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