Practical Pollination Biology

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Protocol 3.9 Pollen staining and fixation with gelatine-fuchsin (Beattie 1971a)

Materials

- 175 mL distilled water
- 150 mL glycerine
- 50 g gelatin
- 5 mg crystalline phenol (Sigma P 3653)
- basic fuchsin crystal (Eastman Fine Chemicals, No. 1762C)
- nail varnish
- alcohol heater or lighter

Method

1. Add the gelatin to the distilled water in a large beaker and heat until dissolved. Then add the glycerine and the crystalline phenol and mix gently.
2. Add some crystals of basic fuchsin and mix until the solution is pale pink to pink.
3. Add the rest of the crystals gradually to the pink solution, and stir gently while the solution is still warm.
4. Filter the solution through glass wool and store it in clear uncontaminated containers.
5. When needed, with a scalpel cut a small cube (2 × 2 × 2 mm) of the solid gelatine-fuchsin, melt it gently on a slide add the examined pollen sample, and cover.
6. When the preparation is ready it can be kept in a cool and dark place for 3-4 years after sealing the edges with nail varnish.

Note: Do not overheat the melted gelatin as it could spoil the mountant. This technique can be applied to a whole stigma (to evaluate the pollen dose) or directly to an insect or other animal to identify and count its pollen load. Phenol is toxic, handle with great care.

Protocol 3.10 Pollen staining and fixation, modified Calberla's solution (Ogden *et al.* 1974; P. Bernhardt, pers. comm.)

Materials

- glycerine

- 10 mL 95% ethanol
- 15 mL distilled water
- crystals of aqueous basic fuchsin (mixed with enough water to make a thick slurry or supersaturated solution)
- melted glycerine-jelly (optional)
- nail varnish

A. *Preparation of the Calberla's solution*

1. Mix the first three ingredients. Filter the supersaturated solution of aqueous basic fuchsin through Whatman No. 1 filter paper and reserve the dark purple liquid. Add the purple liquid drop by drop to the solution until the fuchsin turns a pale, transparent pink. Do not allow the stain to become a dark ruby or claret red!
2. Add 2-3 drops of the melted glycerine-jelly. This may dilute the basic fuchsin to the point where more basic fuchsin must be added to restore the pale pink tint. Store at room temperature.

B. *Use*

1. Place the pollen grains on a slide, add 2-3 drops of the Calberla's solution directly on the top of the pollen grains and wait 7-10 min before placing the cover slip.
2. Most pollen can be identified immediately after placement of the cover slip or it can wait until the following day.
3. Glycerine-jelly causes the solution to act as a semipermanent mount. The edges of the cover slip can be sealed with nail varnish and the slide may be stored in a cool dark place for 3-4 years.
4. To remove pollen from a dead insect 'wash' it gently with 2-3 drops of absolute ethanol above the surface of a slide, remove the insect, let the ethanol evaporate, and place the Calberla's solution as in Step I.
5. Viewing of the slide is best 24 h later when the colouring is deep enough for recognition of details. The wall of the pollen is stained in pink-red, showing the pores, colpi, and exine sculpture. The pollenkitt does not stain and its contrasting pigmentation, if present, can be seen on the exine surface as a series of oil droplets.

Note: If the solution is too red it stains both the pollen and the cytoplasm. This obscures pores, colpi, and opercula, which are needed for final identification. A light pink solution stains *only* layers

Protocol 3.16 DAB test for peroxidases presence in pollen (Rodriguez-Riaño and Dafni 2003)

Materials

- Sigma Fast™ 3.3' diaminobenzidine (DAB) tablets set (Sigma D-4168)
- 1 mL distilled water
- vial (1-5 mL) with a screw cap
- glycerine
- nail varnish

Method

1. Dissolve one "golden" and one "silver" tablet in 1 mL distilled water in the vial.
2. Wait 5 min and shake the solution.
3. Put the pollen sample in a 5-10 µL droplet of the reagent on a slide. Mix it well to prevent clumps. Let it dry and repeat the procedure. Be careful not to use too much pollen, or the reagent may not reach individual grains. When the second application of the reagent is dry, add a droplet of glycerine or water and seal the preparation with nail varnish.

4. Examine the preparation under the microscope. A dark brown-purple-red indicates presence of peroxidases. The stain lasts for at least 4-5 days.

Note: Be careful to work above 20°C to enhance the reaction. The preparation can be warmed gently with a lighter. Do not touch the reagent with bare hands. To prevent environmental contamination, use plastic vials and rinse them off in absolute ethanol before burning them after use. The reagent is active for up to about 24 h after preparation. The reagent may indicate, in the same preparation, the receptivity of the stigma as well as the viability of the pollen grains that reached it.

Protocol 3.17 MTT test for presence of dehydrogenases in pollen (Rodriguez-Riaño and Dafni 2000)

Materials

- 100 mg MTT (3-4.5 Dimethylthiazol-2-yl-2.5-diphenyl-tetrazolium bromide, Sigma M-2128)
- Whatman No 1 filter paper
- 5 mL sucrose 5%
- glycerine or distilled water
- nail varnish

Method

1. Dissolve the MTT in the sucrose solution filter paper, put back in the original bottle and strain through Whatman No 1. If possible, keep it between 2 and 8°C.
2. Put the pollen sample in a 5-10 µL droplet of the reagent on a slide, mix well, let it dry, and repeat the procedure.
3. Be careful not to use too much pollen, as the reagent may not penetrate the individual grains.
4. To enhance the reaction, the procedure should be at a temperature above 20°C. Heat the preparation gently with an alcohol lamp or lighter if needed to warm the preparation.
5. Add a small droplet of glycerine to the stained dry sample, seal the preparation with nail varnish and check under the microscope. Dark violet-purple-brown pollen indicates the presence of dehydrogenases.

Protocol 3.19 Pollen germination *in vitro*

Materials

- 3 cm Petri dishes (or multiwell plate, *e.g.* Sigma M 9655)
- sucrose solutions (0, 5, 10, 20, 30, 40, 50, 60%) as percentage by weight (g sucrose/100 g solution)
- 2×10^{-3} M H_3BO_3 (2 mg in 1 L of distilled water)
- 6×10^{-3} M $Ca(NO_3)_2$ (6 mg in 1 L of distilled water)
- hand refractometer (customized for small samples, *e.g.* Stanley and Bellingham's model 45-5 for sugar concentration range of 0 to 50% and model 45-05 for a range of 45 to 80%.)
- Vaseline
- methylene blue (1% in water) or methylene green/phloxine (*Protocol 3.13*)

Method

1. Prepare the suitable sucrose concentration stock in a 1:1 mixture by volume of the solutions of H_3BO_3 and $Ca(NO_3)_2$. Check the sucrose concentration in a refractometer prior to the experiment.
2. Mark 4 × 4 squares on the outer sides of 3 cm Petri dish covers. Number the grids prior to adding the droplets. (The number of the dishes depends on how many replicas and solutions are used.)
3. Put a 10 μL droplet of the test solution on to the center of each square in the inner side of the dish cover.
4. Add fresh pollen to each droplet (note: do not apply too much pollen - about 200 grains per droplet is sufficient). Each row can be used for a different plant or population or species.
5. In the bottom plate put 2 mL of solution of the same sucrose concentration as that which is on the inner side of the top plate. This keeps humidity in the chamber in equilibrium, so the concentration in the drops on the inner cover remains more or less constant.
6. Smear the edges of the bottom plate with enough Vaseline to seal the two parts.
7. Turn the dish cover gently with the droplets down (the droplets stay in place) over the bottom dish.
8. Repeat the procedure for each sucrose concentration.
9. Leave the plates (at least six replicates per each treatment) under room conditions for 24 h.

10. At the end of the experiment, put all the dishes in the refrigerator (about 0°C) until examination (which is time consuming!).

11. To check the germination rate open the dish, turn the cover and add a little droplet (1-5 μL) of methylene blue to each sample. Then transfer each sample to a slide, cover gently, and count the pollen grains for germination rates, or mount the samples temporarily with nail varnish to prevent evaporation (they keep for several days at room temperature) until counting.

12. If the experiment is to examine the germination dynamics *vs.* time, leave the plates at room temperature or incubate them at appropriate temperature. Check the percentage of pollen germination periodically under the microscope via the closed cover plates or freeze replicates at every given period of time and count later.

13. Express the results as percentage of germination *vs.* concentration for each examined time period.

14. In germination rate experiments, draw the germination course *vs.* time. Generally, an S-shaped curve is obtained. To compare treatments, species, *etc.*, use the following parameters:
    - M - the final percentage of germination
    - S - the start of the germination (of a given species in a medium of given concentration); the time needed to reach a value of 1/6 of the final germination (M) in that particular concentration
    - R - rate of germination, 4/6 of the final germination (M) divided by the time needed for germination rate to be achieved from 1/6 M to 5/6 M

The same procedure of pollen germination (*Protocol 3.19*) can be used to check the pollen germination in salt solutions (*e.g.* of sea-shore plants) or any other liquid. For additional *in vitro* tests and specific requirements for pollen germination see Shivanna and Johri (1985:118-132) and Stanley and Linskens (1974:67-72). This method of examining pollen germination *in vitro* (*Protocol 3.19*) allows minimization of osmotic changes in the solution and simultaneous testing of large samples. It also makes it possible to examine each sample gradually later on and following the germination rate (the samples can also be photographed periodically) without any intervention.

Any opening of the Petri dish for manipulation may cause a sudden change of the micro-environment around the droplets, so changes in their concentration may occur. Differential germination rates in the central zone of the droplet, as against the margin, are overcome by an even dispersion of the whole droplet at the final counting. Stanley and Linskens (1974:73-74) mention several

possible sources of error in germination tests. Examples are pollen density; pollen dispersion in the droplet; aeration; accumulation of metabolic products which may act as germination inhibitors; presence of sodium ions (if tap water or buffer are used as media); improper pH; and use on non-optimal solution.

The classical pollen germination recipe is that of Brewbaker and Kwack (1963):

- 1 L 10% sucrose (can be varied)
- 100 mg H_3BO_3 (boric acid)
- 300 mg $Ca(N0_3)_2 \cdot 4H_20$ (calcium nitrate)
- 200 mg $MgS0_4 \cdot 7H_20$ (magnesium sulfate heptahydrate)
- 100 mg KNO_3 (potassium nitrate)