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APPENDICES

Appendix 1 Average Temperature, precipitation and relative humidity at the Sakaerat Environmental Research Station (SERS) (data from 1997-2002)

Month	Mean Temperature (°C)	Total Precipitation (mm)	Mean Relative Humidity (%)
January	24.4	1.0	86.2
February	25.9	26.3	82.8
March	28.7	37.0	80.4
April	28.8	108.6	83.8
May	28.1	109.8	87.8
June	28.2	61.7	85.2
July	27.3	44.0	85.8
August	28.2	129.7	85.5
September	26.4	214.1	90.3
October	25.0	168.8	93.3
November	23.0	51.3	78.3
December	22.3	2.0	77.1
Average	26.4	79.5	84.7

Appendix 2 Average Temperature, precipitation and relative humidity at the Nang Rong Weather Station, Buri Ram Province, 20 km outside of the Phanom Rung Historical Park (data from 1997-2002)

Month	Mean Temperature (°C)	Total Precipitation (mm)	Mean Relative Humidity (%)
January	24.7	4.2	70
February	26.5	20.0	67
March	28.4	81.7	69
April	29.1	83.8	74
May	28.6	184.1	79
June	28.6	183.9	78
July	28.2	131.8	79
August	27.8	203.7	81
September	27.2	241.8	85
October	26.8	112.4	84
November	25.0	44.8	77
December	23.6	2.0	72
Average	27.0	107.85	76

Appendix 3 Average Temperature, precipitation and relative humidity at the Bangkok Metropolis Weather Station, Bangkok Metropolis (data from 1997-2002)

Month	Mean Temperature (°C)	Total Precipitation (mm)	Mean Relative Humidity (%)
January	28.0	15.1	68
February	28.9	37.7	71
March	29.9	57.2	72
April	30.6	97.9	73
May	30.1	245.5	75
June	29.8	137.4	74
July	29.6	130.6	74
August	29.1	203.5	74
September	28.7	336.7	78
October	28.5	331.6	79
November	28.0	52.0	70
December	27.3	1.8	64
Average	29.0	137.3	73

Appendix 4 Average Temperature, precipitation and relative humidity at the Phatthalung Agroclimatological Weather Station, Phatthalung (data from 1997-2002)

Month	Mean Temperature (°C)	Total Precipitation (mm)	Mean Relative Humidity (%)
January	27.1	150.7	81
February	27.5	175.9	78
March	28.4	135.7	77
April	29.1	93.1	77
May	29.3	115.9	77
June	29.0	94.0	74
July	28.8	87.6	75
August	28.6	104.7	75
September	28.4	124.4	77
October	28.0	215.8	80
November	27.1	554.0	84
December	26.6	461.5	84
Average	28.2	186.4	78

Appendix 5 Preparation of plant sample for SEM study
(Adapted from Nopanitaya, 1987).

1. Sample Preparation

1.1 Specimen collection and special consideration

The practical steps used in preparing tissues for SME study.

1. If possible, rinse the surface of specimen prior to collection.
2. Fix in 2.5% glutaraldehyde or 4% paraformaldehyde at 4 ˚C for 6-12 h.
3. Wash in appropriate buffer for 15-20 minutes, twice.
4. Trim or select the specimen.
5. Post-fix in 1% Osmium tetraoxide for 1 - 2 hours at room temperature.
6. Pipette out osmium fixative.
7. Start dehydration.

1.2 Dehydration

Dehydration consists of these following steps.

1. Transfer well-fixed and osmicated tissues into 35% ethanol for 15-20 minutes.
2. 50% ethanol 15-.20 minutes.
3. 70% ethanol 15-20 minutes.
4. 95% ethanol 2 changes, 15-20 minutes each.
5. 100% ethanol 3 changes, 15-20 minutes each.
6. Bring the sample to dry by am appropriate procedure.

2. Drying

Method for Critical point drying (CPD)

1. Dehydrate the samples through a grade series of alcohol. .
2. Place samples in the CPD's chamber.
3. Allow the liquid CO_2 to enter the chamber for substituting.
4. Let the temperature and pressure rise up to 30 ˚C and 1,000 psi.

5. Allow standing for 20 minutes.

6. Bleed the gases out

7. Take the dried sample out and keep in the dried dust-free environment.

3 Mounting

Dried samples are needed to adhere to stub, this process is known as mounting. Before mounting is begun, the dried samples are needed to be oriented the desired surface with dissecting stereomicroscope. Upon selection and orientation of sample, specimen can be adhered to stub with adhesive materials. SME stubs are, generally, materials that have characteristics of electrical conductive. These can be, brass, copper, aluminum etc. Brass stubs are mostly used, since they are inexpensive and easily made. Regardless of stub composition, the stub should be well cleaned prior to use. Adhesives used for adhering the dried samples also have the properties of gluing and electrical conducting. Mounting is done by apply adhesive material on stub's surface, followed by placing dried specimen with right side for viewing upward, let the adhesive to dry.

4. Coating

Most biological specimens conduct electrical current poorly. In addition, they are sensitive to electron beam bombardment resulting in surface's destruction and damage. These problems can be reduced and improved by coating the sample's surface with a thin layer of heavy metal's atoms, this established procedure is coating, it is considered to be very important and essential step prior to examining specimens. Mount samples are coated with atoms of heavy metal such as gold by the use of vacuum evaporator. The basic principle of coating is to ionize the gas molecules so they will bombard the target, which may be gold. The atoms will be released following the bombardment under the condition of high voltage and low pressure, thus they will adhere to the surface forming a thin layers of metal's atoms used. After coating is finished, the examination of the specimens can be done under SME.

Appendix 6 Tetrazolium test (Shivanna and Rangaswamy, 1992)

Tetrazolium test is based on the reduction of a colorless soluble tetrazolium salt to a reddish insoluble substance called formazan, in the presence of dehydrogenases.

Special Requirements

2,3,5-triphenyl tetrazolium chloride (TTC) or and other suitable tetrazolium salt

Prepare 0.2-0.5 % TTC in sucrose solution of suitable concentration, to prevent bursting of pollen grains. Addition of a small amount of triazine derivatives to TTC solution in sucrose acts as an intermediate co-factor and thus facilitates of hydrogen ions by the tetrazolium salt and consequently improves the pollen response.

(**Precaution**: TTC solution undergoes photo-oxidation; hence store it in a brown bottle; TTC solution can be stored in the refrigerator for a couple of weeks.)

Procedure

1. Take a drop of TTC solution on a microslide.
2. Suspend a small amount of pollen in the TTC drop and distribute it uniformly in the drop.
3. Apply a coverglass.

(**Note**: oxygen inhibits reduction of TTC, and therefore hanging drop or sitting drop cultures are not suitable.)

4. Transfer the preparation to a humidity chamber (> 95% RH).
5. Incubate the preparation in dark (keep the preparation in a closed chamber such as table drawer) under laboratory temperature or at 30 $\pm$ 2°C for 30-60 min.
6. At the end of the chosen incubation period, observe the preparation under microscope and score it for percentage of viable pollen grains, i.e., pollen grains that have turned red due to accumulation of formazan.

(**Precaution:** score pollen grains from only the central area in the preparation; pollen laying near the margin of the coverglass show variable degrees of red coloration due to higher oxygen availability.)

Appendix 7 Culture on Cellophane Membrane (Shivanna and Rangaswamy, 1992)

Special Requirements

Culture medium: Brewbaker and Kwack's Medium

Sucrose	10%
Boric acid	100 mg/l
Calcium nitrate	300 mg/l
Magnesium sulfate	100 mg/l
Potassium nitrate	100 mg/l

Cellophane membrane, Whatman filter paper, humidity chamber

Procedure

1. Cut 1-cm2 pieces of cellophane membrane and Whatman filter paper.

2. Place a piece of the membrane between two filter paper pieces and staple the three pieces at one margin.

3. Soak the stapled unit in the culture medium for about 1 h.

4. Remove the soaked unit in from the culture medium and place it on a microslide. Remove carefully the upper piece of the filter paper to expose the membrane.

(**Note**: retain the lower piece of filter paper for providing the culture medium.)

With a dry piece of filter paper, gently blot excess medium on the exposed surface of the membrane.

5. Sprinkle pollen grains uniformly on the membrane.

6. Transfer the microslide (with the pollen culture) into a humidity chamber (>90% RH), and maintain it at 22 $\pm$ 2°C or laboratory conditions.

7. After the desired interval, detach the membrane with forceps from the filter paper piece and place it on a dry microslide. Add a drop of suitable fixative (if the culture is to be scored later, add a drop of glycerine also); lower a coverglass. Instead of adding glycerine, the culture can be sealed with paraffin wax or nail polish.

8. Score the culture for percent pollen germination and average pollen tube length.

Appendix 8 Cytochemical localization of esterases on stigma surface (Shivanna and Rangaswamy, 1992)

Special Requirements

α–Naphthyl acetate, fast blue B, phosphate buffer (0.15 M, pH 6.8), acetone, Pasteur pipets

Procedure

1. Collect fresh, unpollinated but fully developed flowers of selected systems, some representing wet and some dry types of stigma.

2. Handle the flowers at their pedicels with forceps; without injuring the stigma and style, carefully excise the pistil. Use one or two pistils, keep the pistils on a dry microslide chamber (i.e. Petri dish lined with moist paper).

3. Prepare the following reaction solutions in screw-cap bottles.

	Solution A (with substrate)	**Solution B** (control, i.e., without substrate)
α–Naphthyl acetate[a]	5 mg	0 mg
Phosphate buffer 0.15 M[b]	10 ml	10 ml
Sucrose (as osmoticum)	10-15%	10-15%
Fast blue B	25 mg	25 mg

[a] α–Naphthyl acetate is insoluble in phosphate buffer; therefore, first dissolve it in a few drops of acetone in a screw-cap bottle, then add the buffer, sucrose, and fast blue B, and mix thoroughly.

[b] Preparation of buffer: solution X–0.15 M NaH_2PO_4 (i.e., 20.85 g/l), solution Y-0.15 M $Na_2HPO_4.7H_2O$ (i.e., 40.24 g/l) or $Na_2HPO_4.12H_2O$(i.e., 53.78 g/l). Mix 51 ml of solution X with 49 ml of solution Y to get 100 ml of 0.15 M phosphate buffer.

(**Note**: (a) the solutions should be prepared shortly before using them, (b) scrupulously avoid contamination of one solution with the other through glassware and/or tools.)

4. Take a few drops of solution A on a microslide, and solution B on another. It the stigma is massive, a cavity slide or a watch glass may be more convenient than a microslide for incubating the stigma.

5. By keeping the stylar region raised on a couple of superimposed microslides, leave only the stigma (two or three) dipped in each solution.

(Note: avoid bring the cut end of the style/pistil in contact with the solution.)

6. Incubate the preparations in both solutions at 25-30°C in a humid chamber for 10 – 20 min.

(Note: the incubation period varies with the system, and affected by ambient temperature. A good indication of adequate incubation is when the stigmas in solution A turn pinkish/just reddish).

7. From each solution remove the pistils separately and rinse thoroughly with the phosphate buffer in watch glasses/Petri dishes.

8. Make wholemount preparations of the stigmas in glycerine. If the stigma is massive, slice it longitudinally or cut free-hand longitudinal sections and mount in glycerine.

9. Observe the preparations under the microscope. Compare the details of the stigmatic surfaces incubated in solution A and B.

10. Suitably illustrate the preparations.

Appendix 9 Multiple staining for localizing pollen tubes in the pistil

(Adapted from Shivanna and Rangaswamy, 1992)

Special Requirements

1. Staining mixture

Lactic acid	78 ml
Malachite green (1% aqueous)	4 ml
Acid fuchsin (1% aqueous)	6 ml
Aniline blue (1% water soluble)	4 ml
Orange G (1% in 50% ethanol)	2 ml

The above constituents are mixed in the order mentioned and the mixture is stored in amber bottles. It is convenient to prepare 1% stock solution of each of the four stains (malachite green to orange G), and make the final staining mixture afresh.

2. Clearing and softening mixture

Lactic acid	78 ml
Phenol	10 ml

Orange G (1% in 50% ethanol) 2 ml

3. Mounting medium

Lactic acid 50 ml

Glycerol 50 ml

Procedure

1. Fix pistils in modified Carnoy's mixture (absolute ethanol: chloroform: glacial acetic acid 6: 4: 1) for 12 h.

2. Transfer the fixed pistils to water through a descending series of ethanol (70, 0,30,10%), and finally to a few ml of the staining mixture.

(Note: if the pistils are large and fleshy, make one or two slits along the style or slice them longitudinally to facilitate the uptake of stains.)

3. Incubate the pistils in the staining mixture at 45±2°C for 24h.

4. Transfer the stained pistils to the clearing and softening mixture and maintain it for 48 h at 45±2°C.

5. Transfer the softened material to fresh clearing and softening mixture and hydrolyzes in a hot air oven at 58±1°C for 30 min.

(**Note**: if required, the pistils can be stored in lactic acid for later studies. On prolonged storage, the pistils may lose the stain, but not the pollen tubes.)

6. Wash the material twice in lactic acid.

7. Mount the material in the mounting medium and apply gentle pressure to spread the tissue. Pollen tubes stain deep blue and the stylar tissue light green to greenish blue.

(**Note**: if differential staining is not required, omit both malachite green and orange G from the staining mixture.)

Appendix 10 Aniline blue fluorescence method (Adapted from Dafni, 1992)

Special Requirements

FPA solution (formalin 40%, concentrated propionic acid, 50% ethanol, 5: 5: 90 by volume)

8 N sodium hydroxide

0.1 N potassium acetate

Aniline blue

Fluorescence microscope with UV filter

Procedure

1. Fix the excised stigma and style in FPA for 24 h and then store in ethanol 70%. Wash in running tap-water before the next stage.

2. Soften the style for 5 h (depending on the species: the correct amount or time needed for softening can vary between 1 to 12 h) in sodium hydroxide. Rinse in tap-water for 1-3 h to remove the sodium hydroxide.

3. Stain with 0.1% aniline blue in potassium acetate for 4 h.

4. Squash the stained style under a cover-slip and observe under a fluorescence microscope equipped with a filter set (of maximum trans-mission 365 nm). Both pollen tube walls and the callose plugs should show a distinct bright yellow to yellow-green fluorescence.

Appendix 11 Estimation of pollen grain number per flower

(Adapted from Dafni, 1992)

Special Requirements

70% ethanol

Detergent

0.5% methylene blue

Procedure

1. Squash a ripe anther (just before pollen exposure) into 0.9 ml ethanol + 3 drops of dye + 4 drops of detergent.

2. Transfer the squashed anther (carefully rinse the forceps and the blade in the droplet to prevent loss of material) into a calibrated tube and fill up to 1 ml with the same ethanol/detergent solution.

3. Shake well (or stir the suspension with a vortex mixer for 60-90 sec.)

4. Transfer six separate samples of 1 µl each and cont all pollen grains (this can be done with a haemacytometer).

5. To obtain the total number, calculate the average number of the pollen grains per anther based on the dilution factor, and multiply by their number in the flower.

BIOGRAPHY

Mr. Boonsanong Chourykaew was born on the 4th August 1960. He got his Bachelor's Degree of Education (Biology and Health Education) from Chulalongkorn University in 1983, and received his Master's Degree of Science in Biological science (Biology) from Prince of Songkla University in 1991.