APPENDICES

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APPENDIX A

Stomatal size measurement (Aiamla-or, 2005)

Ocular micrometer and stage micrometer (Figure A-1) were used in measuring the size of stomata. The size of magnified objects were measured by a scale of 50-100 divisions on an ocular micrometer. This micrometer was calibrated by a stage micrometer which is a microscope slide with a finely divided scale marked on the surface that calibrate the reticule and improve the accuracy of measurements. Stage micrometer obtains a line capacity as a division of other full 100 divisions, each division is 10 microns or 0.01 mm long. This micrometer was used in benchmarking the width of each division on occular micrometer.

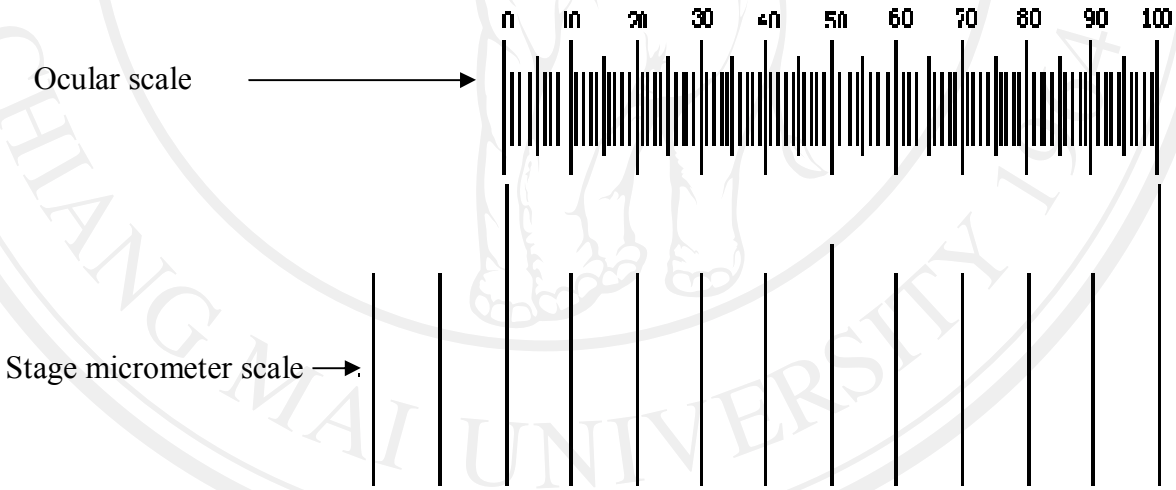

Figure A-1 Scales on ocular and stage micrometers

Stomata featuring on particular epidermis of *Hippeastrum* organs were measured by above-mentioned micrometers, following the procedure suggested by Aiamla-or (2005) as follows:-

1. place a stage micrometer on the microscope stage, using the lowest magnification (4X) and focus on the grid of the stage micrometer

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2. rotate the ocular micrometer by turning the appropriate eyepiece, move the stage until the lines of which the ocular micrometer upon those of the stage micrometer superimpose with the lines of which the two micrometers coinciding at one end of the field, count the spaces of each micrometer to a point at which the lines of the micrometers again coincide
3. calculate the number of micrometers in each space of the ocular scale
4. measure and calculate the size of the stomata as indicated in Figure A-2

Calculation is made, following the equation stated below:-

$$\text{1 division of ocular micrometer } (\mu m) = \frac{\text{no. of stage micrometer that lines on both the reticule x 10}}{\text{no. of stage micrometer that lines on both the reticule}}$$

* 1 μm = 1/1,000 mm

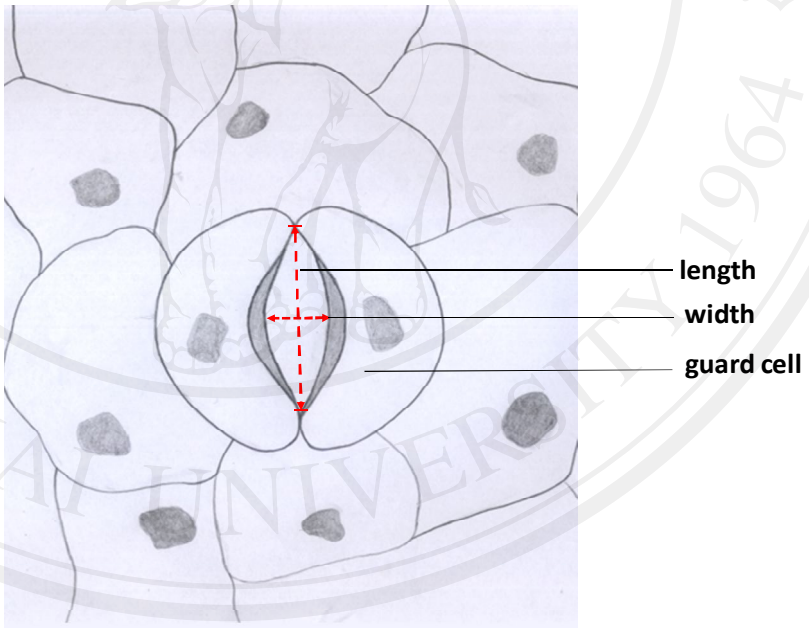

Figure A-2 Stoma of *Hippeastrum*

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APPENDIX B

Stomatal counting via micrometer (Wiriyacharee, 2002)

Procedure of stomatal counting is as follows:-

1. focus the reticular eyepiece, adjust the focus until the lines on the eyepiece are sharp
2. load the stage micrometer under the microscope and focus until the field splits
3. determine the diameter length of each region by counting the numbers of divisions then multiply by 0.01, e.g. the D division has a diameter equal to 0.01D mm

$$\text{microscopic field } (mm^2) = \pi[(0.01D)/2]^2$$

4. determine the area of the microscope field for each objective magnification then make a note
5. place a prepared slide of an epidermal layer on the microscope and focus on the slide using the 10X objective, count the number of stomata within the microscopic field area and take the record
6. average the results and calculate the number of stomata per mm^2

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APPENDIX C

Histological preparation

Botanical microtechnique via paraffin embedding (Johansen, 1940; Sass, 1966)

I Reagent preparation

1. Killing and fixation solution

FAA or Formalin-Acetic acid-Alcohol solution contains

95% ethyl alcohol	50	ml
glacial acetic acid	5	ml
formalin	10	ml
distilled water	35	ml

2. Dehydrating solution

Table C-1 Dehydrating solution

Solvent proportion	Concentration of alcohol				
	50%	70%	85%	95%	100%
95% ethyl alcohol	40	50	50	45	-
absolute alcohol	-	-	-	-	25
tertiary butyl alcohol	10	20	35	55	75
distilled water	50	30	15	-	-

3. Adhesive solution

stock solution :	albumin	1	ml
	distilled water	49	ml

when use, dilute 1 ml of the stock solution with distilled water to 50 ml

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4. Stain

Table C-2 Compositions of Delafield's Hematoxylin, Safranin and Fast Green

stain	chemicals	
Delafield's hematoxylin	aluminium sulfate ($Al_2(SO_4)_3.16H_2O$)	400 ml
	hematoxylin ($C_{16}H_{14}O_6$)	4 g
	95% ethyl alcohol	25 ml
	methyl alcohol	100 ml
	glycerol	100 ml
Safranin	safranin O ($C_{20}H_{19}N_4Cl$)	4 g
	methyl cellosolve	200 ml
	95% ethyl alcohol	100 ml
	sodium acetate	4 g
	formalin	8 ml
	distilled water	100 ml
Fast Green	fast green FCF ($C_{37}H_{34}N_2O_{10}S_3Na_2$)	0.15 g
	methyl cellosolve	100 ml
	absolute ethyl alcohol	100 ml
	clove oil	100 ml

Π Methods

1. Collection and subdividing of plant material

- collect and subdivide each specimen to a proper size

2. Killing and fixation (to preserve the cells, avoiding further changes)

- kill and fix the specimens in FAA
- place specimens, not many in number, in a vial with a lid to protect evaporation of solution
- suck the air bubbles out of the tissues to let the solution throughly penetrate into the tissues
- fix the specimens for 18-24 hours or 1-2 weeks, depending upon the tissue structure
- keep the specimens at room temperature

3. Dehydration

Solution for dehydration consists of a mixture of alcohol, i.e. 95% ethyl alcohol, absolute ethyl alcohol and tertiary butyl alcohol (TBA). Ingredient ratios of alcohol concentration is yet shown in the Table C-1.

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Dry erythrosin dye is added to the last solution in the amount enough to give a red tinge to stain the specimens superficially for easy orientation of the objects during embedding and microtoming. The dye will readily come out of the sections after they are brought down to alcohol when the staining is being carried out.

- slowly dehydrate the specimens in a series of alcohol/water solutions of increasing strengths, each for overnight, to remove all the water
- dehydrate the specimens with 50%, 70%, 85%, 95% and 100% alcohol, successively, each for 24 hours
- shift the specimens to TBA 2-3 times, each of 12 hours after the 100 % solution

4. Infiltration

Melted paraffin is allowed to penetrate into the tissues until the tissues are filled with paraffin.

- transfer the specimen to a mixture of equal parts of liquid paraffin and tertiary butyl alcohol reagent at the ratio 1:1, each for overnight (Figure C-1)
- take out the specimens and put in melting paraffin (Paraplast Plus®), incubate at 56°C for 2-4 weeks or until fully inserted.

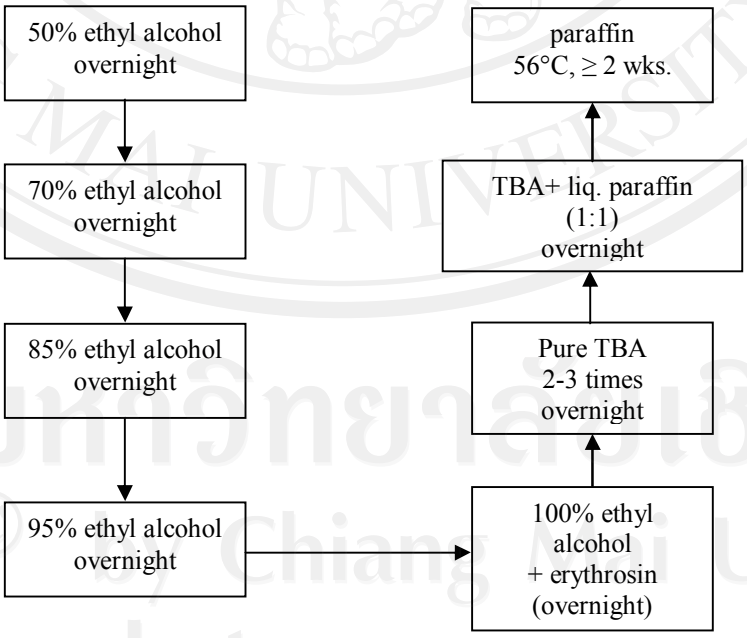

Figure C-1 Dehydrating protocol
(Modified from Kermanee, 2008; Johansen, 1940; Sass, 1966)

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5. Embedding

- embed the specimens by pouring melted paraffin into the mold
- remove air bubbles with a needle heated slightly in the flame
- place specimens in and quickly dispose the pieces of specimens into an orderly arrangement
- leave the mold at room temperature until it cools down.

6. Sectioning

- trim the paraffin block containing single embedded specimen
- stick the block onto the wood bar saturated with paraffin
- fix the wood bar to microtome stage for sectioning
- cut the sections with the thickness of 13-15 μm

7. Affixing

- drop adhesive solution (albumin) onto a glass slide then carefully transfer the specimen ribbon with a scalpel or paintbrush onto the glass slide and arrange to the right position
- examine the sections under the light microscope
- transfer selected slides onto the warming plate, warm them at 40°C until dry

8. Staining

- remove paraffin from the sections in xylene
- stain the sections with a single stain of hematoxylin or with double stain of safranin and fast green, following the staining chart suggested in Figure C-2 and C-3, allow the slide to immerse in each staining jar for 3-5 minutes

9. Mounting

- put a drop of Canada balsam or Permount over the objects on the slide and place a cover glass on top of it
- remove air bubbles off the mounting fluid before closing the cover glass
- leave it at room temperature until dry
- study and observe the specimens under the microscope and photograph

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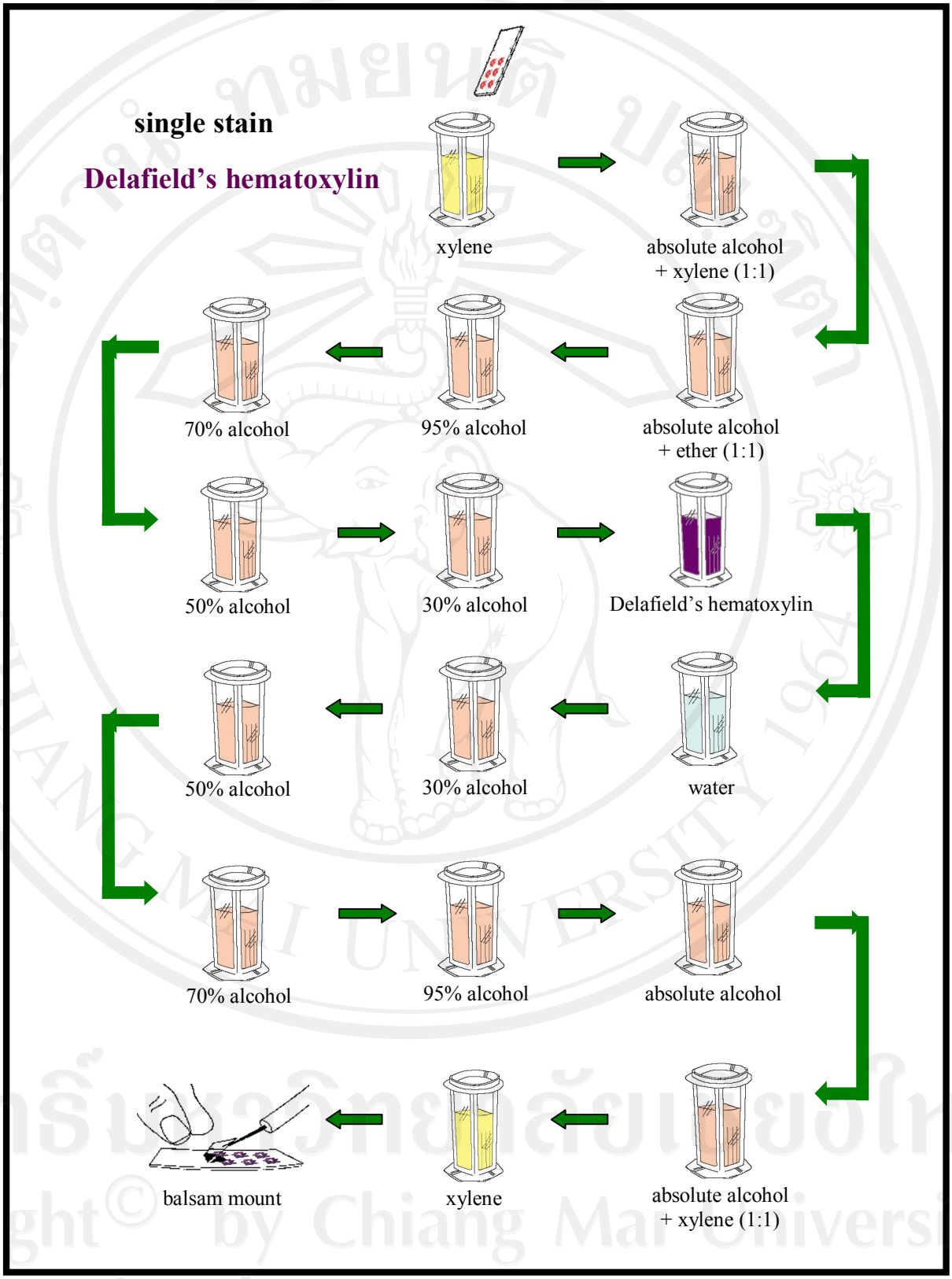

Figure C-2 Protocol for Delafield's hematoxylin staining
(Modified from Kermanee, 2008; Johansen, 1940; Sass, 1966)

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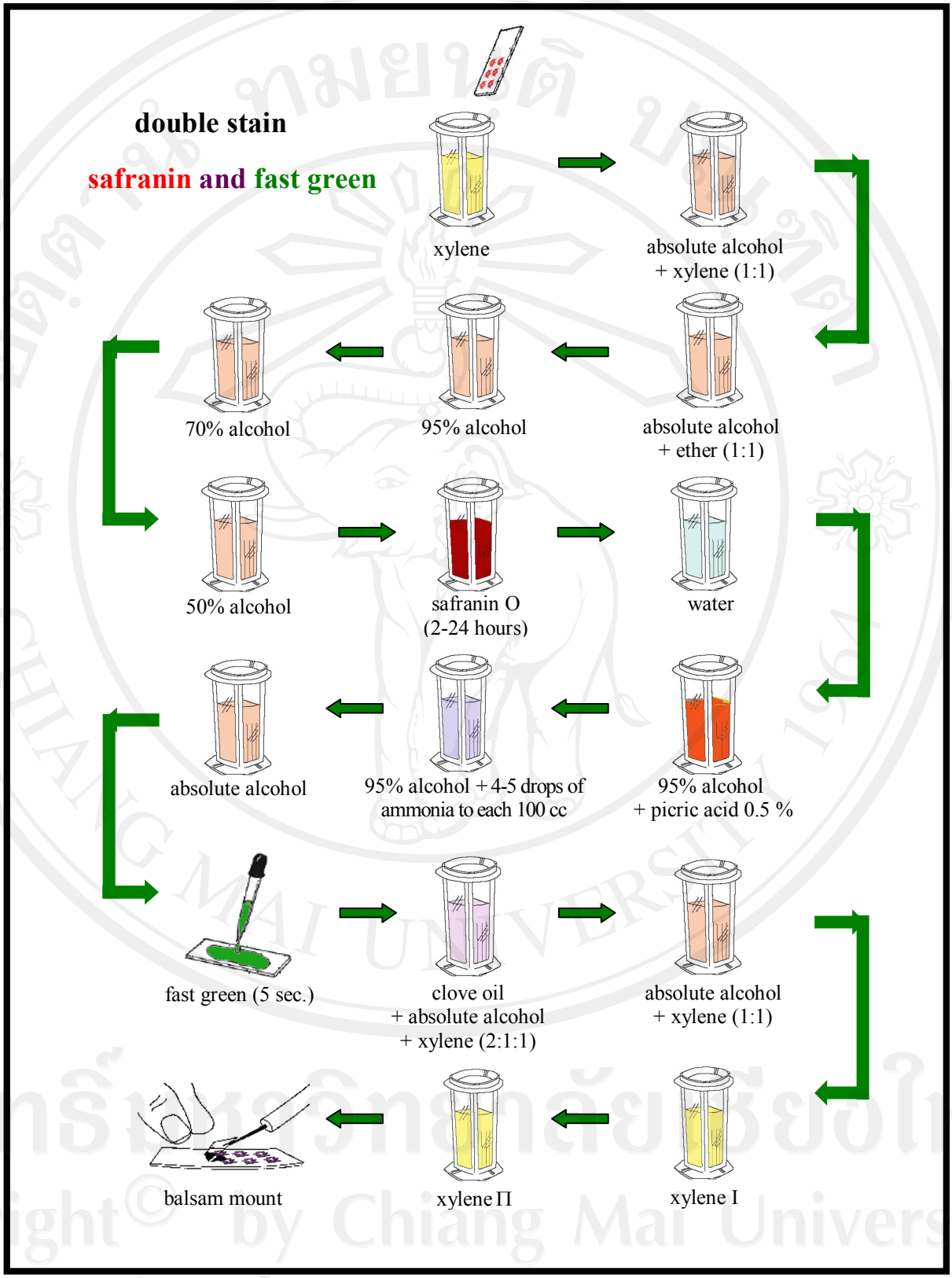

Figure C-3 Protocol for safranin and fast green staining

(Modified from Kermanee, 2008; Thahanthai, 2010; Johansen, 1940; Sass, 1966)

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APPENDIX D

Preparation of carbol fucshin dye

Preparation of carbol fuchsin staining solution were conducted based on Chen's recipe (1992) with a slight modification to suit *Hippeastrum* tissues, as follows:

Fluid A: dissolve 3 g of basic fuchsin in 10 ml of 70% ethanol then add 90 ml of 5% phenol

Fluid B: mix 6 ml of glacial acetic acid and 6 ml of 37% formaldehyde into 55 ml of fluid A

The fluid B, 5-10 ml, was mixed with 95 ml of 45% acetic acid and 1.8 g sorbital then stirred with magnetic stirrer for several minutes.

Fresh carbol fuchsin stain fluid was ready after 30-45 days of incubation.

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APPENDIX E

Statistical analysis

Table E-1 Analysis of variance of number of bulb scale in various bulb sizes

Source	df	SS	MS	F	P
Treatment	1	97002.604	97002.604	116597.454	0.000
Variety	3	79.813	26.604	31.978	0.000
Size	5	2559.721	511.944	615.359	0.000
Var. x Size	15	23.162	1.544	1.856	0.029
Error	216	179.700	0.832		
Total	240	99845.000			

CV = 4.54% LSD = 0.80

Table E-2 Analysis of variance of number of dry flower bud in various bulb sizes

Source	df	SS	MS	F	P
Treatment	1	429.338	429.338	1647.192	0.000
Variety	3	289.446	96.482	370.162	0.000
Size	5	95.937	19.187	73.615	0.000
Var. x Size	15	55.979	3.732	14.318	0.000
Error	216	56.300	0.261		
Total	240	927.00			

CV = 38.20% LSD = 0.45

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Table E-3 Analysis of variance of number of fresh flower bud in various bulb sizes

Source	df	SS	MS	F	P
Treatment	1	620.817	620.817	2056.693	0.000
Variety	3	296.983	98.994	327.957	0.000
Size	5	130.433	26.087	86.422	0.000
Var. x Size	15	14.567	0.971	3.217	0.000
Error	216	65.200	0.302		
Total	240	1128.000			

CV = 34.17% LSD = 0.48

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Arayakitcharoenchai, P. and C. Suwanthada. 2009. Pollen Morphology of *Amaryllis*. p. 59. *In* Proceeding of 8th National Horticulture Congress. 6-8 May, 2009, Chiangmai, Thailand. 402 p.

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