APPENDICES
Appendix A

Histological preparation

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

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>
<thead>
<tr>
<th>Solvent proportion</th>
<th>Concentration of alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>95% ethyl alcohol</td>
<td>40</td>
</tr>
<tr>
<td>absolute alcohol</td>
<td>-</td>
</tr>
<tr>
<td>tertiary butyl alcohol</td>
<td>10</td>
</tr>
<tr>
<td>distilled water</td>
<td>50</td>
</tr>
</tbody>
</table>

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

4. Stain

<table>
<thead>
<tr>
<th>Stain</th>
<th>chemicals</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delafield’s hematoxylin</td>
<td>aluminium sulfate (\text{Al}_2(\text{SO}<em>4)</em>{3.16\text{H}_2\text{O}})</td>
<td>400 ml</td>
</tr>
<tr>
<td></td>
<td>hematoxylin ((\text{C}<em>{16}\text{H}</em>{14}\text{O}_6))</td>
<td>4 g</td>
</tr>
<tr>
<td></td>
<td>95% ethyl alcohol</td>
<td>25 ml</td>
</tr>
<tr>
<td></td>
<td>methyl alcohol</td>
<td>100 ml</td>
</tr>
<tr>
<td></td>
<td>glycerol</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Figure A-1 Protocol for Delafield’s hematoxylin staining
(Modified from Kermanee, 2008; Johansen, 1940; Sass, 1966)
Appendix B

Preparation of carbol fuchsin dye

Preparation of carbol fuchsia staining solution were conducted based on Chen’s recipe (1992) 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 fuchsia stain fluid was ready after 30 - 45 days of incubation.
Appendix C
Chemical reagent preparation for isozyme pattern investigation

1. extraction buffer (0.2M Tris buffer pH 8.4)
   - 0.2M tris 75,000 ml
     (tris 2.42 g + distilled water add to 100 ml)
   - 0.2M HCl 24,750 ml
     (37% HCl 1.7 ml + distilled water add to 100 ml)
   - distilled water add to 300,000 ml
   - pH adjust to 8.4 (with 0.2M HCl)

2. electrode buffer
   - tris 3.000 g
   - glycine 14,400 g
   - distilled water add to 500,000 ml
   - pH adjust to 8.3
     (when use, electrode buffer : distilled water = 1 : 4)

3. marker dye solution
   - bromophenol blue 0.050 g
   - glycerol 1.000 ml
   - tris-HCl buffer pH 6.7 10,000 ml
     (1N HCl 48 ml + tris 5.98 g)
   - marker dye solution : enzyme sample = 1 : 9
4. stock solutions of acrylamide gel

(1) acrylamide/Bis

- acrylamide: 29.200 g
- N,N’-methylene bisacrylamide: 0.800 g
- distilled water add to: 100,000 ml

(2) 1M tris-HCl pH 8.8

- 1M tris: 50,000 ml
  (tris 12.11 g + distilled water add to 100 ml)
- 1M HCl: 8,000 ml
  (37% HCl 8.35 ml + distilled water add to 100 ml)
- pH adjust to 8.8 (with 1M HCl)

5. acrylamide gel 7.5%

- acrylamide/Bis: 10,000 ml
- 1M tris-HCl pH 8.8: 10,000 ml
- distilled water: 19,400 ml
- 10% ammonium persulphate: 400,000 µl
- TEMED: 20,000 µl

6. stock solutions of enzyme staining

(1) 0.1M tris-HCl (for GDH, GOT, SKD)

- tris: 12.110 g
- 37% HCl 8.35 ml + distilled water add to: 100,000 ml
- distilled water add to: 1,000,000 ml
- pH adjust to 7.5 for GDH, and 8.0 for SKD
(2) 0.2M tris-HCl pH 8.0 (for MDH)
tris 2.420 g
distilled water add to 100,000 ml
pH adjust to 8.0 (with 1N HCl)

(3) 0.2M tris-maleate pH 6.0 (for LAP)
tris 2.420 g
maleic acid 2.320 g
distilled water add to 100,000 ml
pH adjust to 6.0

(4) 0.1M Tris buffer pH 4.0 (for POX)
tris 0.377 g
acetic acid 0.400 ml
distilled water add to 250,000 ml
pH adjust to 4.0

(5) 0.2M acetate buffer pH 4.8 (for ACP)
0.2M acetic acid 40,000 ml
(acetic acid 1.15 ml + distilled water add to 100 ml)
0.2M sodium acetate 60,000 ml
(sodium acetate 2.72 g + distilled water add to 100 ml)
distilled water add to 200,000 ml
pH adjust to 4.8
(6) **0.2M phosphate buffer pH 6.0 (for EST)**

- 0.2M monobasic potassium phosphate 87.800 ml
  (monobasic potassium phosphate 2.72 g + distilled water add to 100 ml)
- 0.2M dibasic potassium phosphate 12.300 ml
  (dibasic potassium phosphate 1.74 g + distilled water add to 50 ml)
- distilled water add to 200.000 ml
- pH adjust to 6.0

(7) **1M L-malate (or 2M DL-malate; MDH substrate)**

- DL-malic acid 5.364 g
- NaOH 3.000 g
- distilled water add to 20.000 ml
- pH adjust to 7.0

(8) **2.5% leucine naphthylamide (LAP substrate)**

- L-leucine-2-naphthylamide · HCl 0.050 g
- distilled water add to 2.000 ml

7. **enzyme staining solutions**

   (1) **acid phosphatase (ACP)**

- 0.2M acetate buffer pH 4.8 200.000 ml
- fast garnet GBC (sulfate salt) 0.200 g
- \(\alpha\)-naphthyl phosphate-sodium salt 0.100 g
- filter in dark

   (2) **esterase (EST)**

A : 0.2M phosphate buffer pH 6.0 200.000 ml
B : fast blue B salt 0.300 g
C : \(\alpha\)-naphthyl acetate (in absolute ethanol 6 ml) 0.006 g
- A + B and then filter in dark, and add C
(3) glucose dehydrogenase (GDH)

0.1M tris-HCl pH 7.5 100,000 ml
D(+)-glucose monohydrate 16,000 g
10% NAD 400,000 µl
10% NBT in methyl alcohol 200,000 µl
10% PMS 40,000 µl

(4) glutamate oxaloacetate transminase (GOT)

0.1M tris-HCl 100,000 ml
α-ketoglutaric acid pH adjust to 7.4 0.100 g
L-aspartic acid 0.200 g
10% pyridoxal-5’-phosphate 40,000 µl
fast blue BB 0.200 g

(5) leucine aminopeptidase (LAP)

0.2M tris-maleate pH 6.0 100,000 ml
LAP substrate 2,000 ml
1M MgCl₂·6H₂O 2,000 ml
fast blue RR 0.100 g

(6) malate dehydrogenase (MDH)

0.2M tris-HCl pH 8.0 100,000 ml
1M L-malate (MDH substrate) 20,000 ml
1% NAD 4,000 ml
1% MTT 2,000 ml
1% PMS 0.400 ml
(7) peroxidase (POX)

A : 0.1M tris buffer pH 4.0 160.000 ml
B : β-naphtol (in acetone 20 ml) 0.058 g
C : 3-amino-9-ethylcarbazole (in acetone 20 ml) 0.084 g
D : 3% H$_2$O$_2$ 200.000 µl
A + B + C in dark, then add D

(8) shikimate dehydrogenase (SKD)

0.1M tris-HCl pH 8.0 100.000 ml
shikimic acid 0.060 g
10% NADP$^+$ 400.000 µl
10% NBT in methyl alcohol 200.000 µl
10% PMS 40.000 µl
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