Appendices

Appendix A

Compositions of CMU_1 medium

1. Macro element	mg/l
$Ca(NO_3)_2.4H_2O$	151
$(NH_4)_2SO_4$	500
KNO_3	525
KH_2PO_4	250
$MgSO_4.7H_2O$	250
2. Micro element (Murashige and Skoog, 1962)	**mg/l**
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.830
$Na_2MoO_4.2H_2O$	0.250
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
3. Organic substance	**mg/l**
Glycine	2.00
Thiamine.HCl	0.25
Pyridoxin.HCl	0.25
Nicotinic acid	0.25
Myo-inositol	100.0
4. Addendum	
Coconut water	150 ml
Sucrose	20 g
Agar	6 g
pH adjust to 5.7	

Appendix B

Histological preparation

Appendix B-1 *Paraffin embedding technique* (Johansen, 1940)

1. Fix the specimen in FAA solution.
2. Dehydrate the specimen respectively with 50%, 70%, 85%, 95% and 100% alcohol for 24 hours.
3. Change the dehydrating reagent to TBA (tertiary butyl alcohol) for 1 day (do this step for 3 times).
4. Change the TBA to TBA : liquid paraffin (1:1) reagent for 1 day.
5. Change to melting paraffin (Paraplast Plus®)
6. Incubate at 60°C for 2-4 weeks or more (according to specimen type).
7. Embed the specimen into a paper boat, remove air bubbles by using hot needle, and also arrange the specimen to a right position. Leave it at room temperature until cool down.
8. Serial section by using microtome at 13 µm thickness.
9. Drop adhesive solution onto a glass slide, carefully transfer the specimen ribbon onto the glass slide and arrange to right position.
10. Warm the glass slide on a hot plate at 40°C until dry.
11. Stain the specimen with hematoxylin dye. (see staining protocol)
12. Cover the slide by dropping a drop of Canada balsam and close with microscope cover glass, and then remove air bubbles.
13. Leave it at room temperature until dry.
14. Observe the specimen under light microscope.

Preparation of reagents

1. FAA solution
   Mix 50% ethanol : glacial acetic acid : formalin at ratio 90:5:5 by volume.

2. Dehydration solution

Solvent porportion	Concentration of alcohol				
	50%	70%	85%	95%	100%
Distilled water	50	30	15	0	0
95% ethyl alcohol	40	50	50	45	0
t-butyl alcohol (TBA)	10	20	35	55	75
Absolute ethanol	0	0	0	0	25

Staining protocol

Immerse the specimen slide into various solutions for 3-5 minutes following these steps:

1. Xylene
2. Xylene : absolute ethanol (1:1)
3. Absolute ethanol : ether (1:1)
4. 95%, 70%, 50% and 30% ethanol, respectively.
5. Hematoxylin dye
6. Tap water for several times
7. 30%, 50%, 70%, 95% and absolute ethanol, respectively.
8. Xylene : absolute ethanol (1:1)
9. Xylene

Appendix B-2 Modified technique for using freezing-microtome

Set up freezing-microtome (CRYO CUT 1800, Reichert-Jung) and then adjust the optimal cutting temperature at -20°C.

Sample preparation

1. Fresh sample or FAA-fixed sample can be used.
2. Longitudinal cut passing the middle of the sample by free-hand cut with super thin Gillette® blade. Observe under a stereo microscope to select the right-side sample.
3. For FAA-fixed sample, rinse the sample with distilled water.
4. Dropping a drop of embedding compound (O.C.T. embedding compound Tissue-Tek® #4583) onto a specimen-holding board (use only for freezing-microtome). Embed the sample by placing upside down the right-side close to the specimen-holding board.
5. Cover the specimen with embedding compound.
6. Carefully press the specimen close to the board and then place into the freezing-microtome chamber. Wait until the specimen is frozen.
7. Carefully cut off the specimen to decrease its thickness until very thin section of the right-side specimen is remained on the board.
8. Melt the specimen at room temperature.
9. Carefully transfer the specimen to a glass slide, rinse off the embedding compound with distilled water and cover with microscope cover glass.
10. Observe the specimen under a light microscope.

The specimen can be stained with a specific dye to observe some metabolite in the studied tissue, e.g. iodine solution for staining starch grain.

The specimen can be kept in FAA solution to be preserved for further study.

Appendix C

Chemical analysis

Appendix C-1 Extraction with 80 % ethanol

The procedure has been described by Associate Professor Dr. Ohtake Norikuni, Niigata University, Japan (personal communication)

A) Protocol for free amino acid analysis

1. Weigh about 50 mg homogenized freeze-dried sample and put into 1.5 ml appendorf tube.
2. Add 500 µl of cooled 80 % ethanol, homogenize with blender and rinse the blender probe with 500 µl of 80 % ethanol, mix well.
3. Incubate in heat box at 60°C for 15 min., and cool down at room temperature.
4. Centrifuge in a refrigerated centrifuge at 4°C, 8,000 rpm for 10 min.
5. Collect the supernatant into a small vial.
6. Wash the residue with 500 µl of 80 % ethanol, vortex to mix well, centrifuge at 4°C, 10,000 rpm for 10 min., and collect the supernatant into the same vial.
7. Wash the residue again with 500 µl of 80 % ethanol, vortex to mix well, centrifuge at 4°C, 12,000 rpm for 10 min., and collect the supernatant.
8. Dry the extract solution in a vacuum evaporator.
9. Add 1,000 µl de-ionize distilled water, and then dissolve well using an ultrasonic generator.
10. Filter the extract solution, pass through 0.45 µm Millipore filter membrane to 1.5 ml appendorf tube.
11. Keep at -20°C.

B) Protocol for total soluble sugar analysis

Modified Ruamrungsri (1997) protocol for tissue culture samples in Experiment 4 and Experiment 5 (Part I)

1. Weigh about 100 mg fresh sample and put into 1.5 appendorf tube.
2. Add 500 µl of cooled 80 % ethanol, homogenize with a blender in an ice box and rinse the blender probe with 100 µl of 80 % ethanol.
3. Incubate in a water bath at 60°C for 15 minutes, cool down at room temperature.
4. Centrifuge at 10,000 rpm for 10 minutes.
5. Collect the supernatant into a new appendorf tube.
6. Wash the residue with 200 µl of 80 % ethanol, vortex to mix well, centrifuge at 10,000 rpm for 10 minutes, and collect the supernatant.

7. Repeat step 6.
8. Adjust final volume to 1,000 µl with 80 % ethanol.
9. Keep the extracted solution at -20°C.
10. Dry the residue in a refrigerator, and keep in a freezer. (for future starch analysis)

Appendix C-2 Extraction with 8.14 N $HClO_4$

Modified Ruamrungsri (1997) protocol for tissue culture samples in Experiment 4 and Experiment 5 (Part I)

1. Use the dried residue from step 10 in appendix III-1B. (dry weight of the residue is calculated from weight of the appendorf tube both before and after step 2)
2. Carefully transfer the dried residue to 10 ml centrifuge tube.
3. Add 0.5 ml de-ionize distilled water.
4. Incubate in a water bath at 100°C for 15 min.
5. Add 0.65 ml of 8.14 N $HClO_4$, vortex to mix well every 5 min., for 20 minutes.
6. Add 2.0 ml de-ionize distilled water and vortex to mix well.
7. Centrifuge at 10,000 rpm for 10 min., collect the supernatant into 10 ml volumetric flask.
8. Repeat steps 5-7.
9. Adjust final volume to 10 ml with 8.14 N $HClO_4$
10. Filter the extract solution with Whatman® No 1 paper filter.
11. Keep at -20°C.

Appendix C-3 The Kjeldahl method (modified by Ohyama *et al.*, 1991)

1. Weigh about 50 mg sample powder, put into a 25x150 mm test tube. (Except in blank preparation)
2. Add 1 ml of conc. H_2SO_4 and gently mix well.
3. Seal the test tube with parafilm, leave it overnight at room temperature.
4. Remove the parafilm and gently mix well.
5. Incubate in a heat box at 180°C for 10 min., and then add 0.3 ml of 30% H_2O_2.
6. Incubate in a heat box at 230°C for 30 min., and then add 0.3 ml of 30% H_2O_2, Repeat this step until the digested solution is clear.
7. Leave it at room temperature until it is cool.
8. Pour the digested solution to 50 ml volumetric flask and adjust to a final volume by filling up with de-ionize distilled water.
9. Keep at room temperature.

Appendix C-4 *The $HClO_4$-HNO_3 method* (modified by Mizukoshi *et al*., 1994)

1. Weigh about 50 mg sample powder and put into a 25x150 mm test tube. (Except in blank preparation)
2. Add 0.4 ml of conc. $HClO_4$ and 0.5 ml of conc. HNO_3, gently mix well.
3. Seal the test tube with parafilm, leave it overnight at room temperature.
4. Remove the parafilm and gently mix well.
5. Incubate in a heat box at 100°C until yellowish-orange vapor is removed.
6. Incubate in a heat box at 200-210°C until the extract solution is clear.
7. Leave it at room temperature until it is cool.
8. Add 1 ml of conc. HCl : H_2O at 1:4, and incubate at 100°C for 5 min. to remove Cl_2 gas.
9. Leave it at room temperature until it is cool.
10. Adjust to a final volume by de-ionize distilled water in 50 ml volumetric flask.
11. Keep at room temperature.

Appendix C-5 *The Phenol-Sulfuric acid assay* (Dubois *et al*., 1956)

1. Add 0.5 ml de-ionize distilled water into a 25x150 mm test tube.
2. Use 10-20 µl extract sample solution and fill up with 80% ethanol to 50 µl final volume. (the same as the used volume of the std. D-glucose; 50 µl, to make a standard curve)
3. Add 0.5 ml of 5% phenol (except in blank reaction, using de-ionized distilled water), gently mix well.
4. Quickly and directly add 2.5 ml of conc. H_2SO_4 and mix well.
5. Leave for 10 minutes and mix well again.
6. Determine absorbance at 485 nm by using a spectrophotometer.

Preparation of standard D-glucose

1. Prepare 1,000 ppm of D-glucose in 80% ethanol.
2. Dilute to make std. 0, 100, 200, 300, 400 and 500 ppm D-glucose.

Appendix C-6 *The Anthrone method* (JSPN, 1990)

1. Use 1.0 ml of diluted 5x - 20x of extract sample solution into a 25x150 mm test tube.
2. Keep in a water bath at room temperature.
3. Quickly and directly add 2.0 ml Anthrone solution, and then mix well.
4. Incubate the test tube in a water bath at 100 °C for 7.5 minutes.
5. Quickly cool down in the water bath at room temperature and then mix well.
6. Determine absorbance at 630 nm by using a spectrophotometer.

Calculation for starch content

Starch concentration (ppm) = Glucose concentration x 0.9

Preparation of standard D-glucose

1. Prepare standard 1,000 ppm of D-glucose (dissolve 100 mg of D-glucose in 100 ml of de-ionize distilled water).
2. Dilute to 100 ppm of standard D-glucose solution.
3. Dilute again to make 0, 10, 20, 30, 40 and 50 ppm of standard D-glucose.

Preparation of Anthrone solution

Dissolve 1 g of anthrone in 500 ml of conc. H_2SO_4. This solution must be prepared before use.

Appendix C-7 *The Indophenol method* (modified by Ohyama *et al*., 1991)

1. Add 0.4 ml digested sample solution into a 25 ml volumetric flask.
2. Add 0.5 ml each of solvent A and solvent B, respectively.
3. Titrate with 1N NaOH until pink color change into yellow.
4. Add 2.5 ml each of solvent C and solvent D, respectively.
5. Adjust to a final volume of 25 ml with de-ionize distilled water.
6. Leave it at room temperature for 3 hr. until it changes into blue color.
7. Determine absorbance at 625 nm by using a spectrophotometer.

Preparation of standard N

1. Prepare standard 100 ppm of N (dissolve 473.9 mg of $(NH_4)_2SO_4$ (assay=99.5%) in 1,000 ml of 0.5N H_2SO_4).
2. Dilute to make 5 ppm standard N.
3. Dilute again to make standard N at 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ppm.

Preparation of solvents

Solvent A

Dissolve 25 g Na_2.EDTA in 800 ml de-ionize distilled water, adjust pH = 10 with 10N NaOH. Add 20 ml methyl red solution (dissolve 0.05 g methyl red in 20 ml of 60% ethanol). Adjust to a final volume of 1,000 ml, and then keep it in the dark.

Solvent B

Separately dissolve 136.09 g KH_2PO_4 and 2.75 g benzoic acid in 400 ml of de-ionize distilled water, and then mix them together. Adjust to a final volume of 1,000 ml.

Solvent C

Dissolve 0.1 g sodium nitroprusside in about 500 ml de-ionize distilled water, and then add 10.25 ml phenol (melt at 40 ºC before use). Adjust to a final volume of 1,000 ml, and then keep it in a refrigerator at 4 ºC. This solution can be kept for 2 weeks.

Solvent D

Dissolve 10 g NaOH, 7.06 g $Na_2HPO_4.7H_2O$ and 31.8 g $Na_2PO_4.12H_2O$ in 500 ml de-ionize distilled water, and then add 10 ml of sodium hypochlorite (assay Cl=5%). Adjust to a final volume of 1,000 ml.

Appendix C-8 The Ammonium molybdate method (modified by Ohyama *et al*., 1991)

1. Add 2.0 ml digested sample solution into a 25 ml volumetric flask.
2. Add 1.0 ml of solvent A.
3. Add 0.2 ml of solvent B.
4. Adjust to a final volume of 25 ml with de-ionize distilled water.
5. Leave it at room temperature for 15 min.
6. Determine absorbance at 660 nm by using a spectrophotometer.

Preparation of standard P

1. Prepare 500 ppm standard P (dissolve 716.5 mg KH_2PO_4 in 25 ml H_2SO_4 solution (dilute H_2SO_4 : distilled water; 5:1).
2. Dilute to make 5 ppm standard P.
3. Dilute again to make standard P at 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ppm.

Preparation of solvents

Solvent A

Dissolve 25 g $(NH_4)_6Mo_7O_{24}$ in 200 ml de-ionize distilled water and carefully fill up with H_2SO_4 solution (dilute 250 ml of conc. H_2SO_4 in a final volume of 500 ml), leave it cool down at room temperature, and then adjust to a final volume of 1,000 ml, keep in the dark.

Solvent B

Weigh 0.25 g of $SnCl_2.2H_2O$ and put into a bottle. Carefully pour 5 ml of conc. HCl in a hood, dissolve well and then fill up with 20 ml de-ionize distilled water, keep in the dark. This solution can be kept for 2 days.

Appendix C-9 Analysis of K, Ca and Mg by using Atomic absorption spectrophotometer (modified by Mizukoshi *et al*., 1994)

Preparation of standard K

1. Use standard 1,000 ppm of K (in 1% HCl).
2. Dilute the standard K to 100 ppm, and to 10 ppm with de-ionize distilled water, respectively.
3. Dilute again to make standard K at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ppm with de-ionize distilled water.

Preparation of standard Ca

1. Use standard 1,000 ppm of Ca (in 1% HCl).
2. Dilute the standard Ca to 100 ppm, and to 5 ppm with La_2O_3 solution.
3. Dilute again to make standard Ca at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ppm with La_2O_3 solution.

Preparation of standard Mg

1. Use standard 1,000 ppm of Mg (in 1% HNO_3).
2. Dilute the standard Mg to 100 ppm, and to 5 ppm of with La_2O_3 solution.
3. Dilute again to make standard Mg at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ppm with La_2O_3 solution.

Preparation of La_2O_3 solution

Dissolve 2.01 g La_2O_3 in 500 ml de-ionize distilled water, add 10 ml conc. HCl and then adjust the final volume to 1000 ml.

Analysis of K

1. Dilute the extract sample solution to make its concentration into the range of standard K (use 0.2-1.0 ml depends on type of plant tissues) to a final volume of 25 ml with de-ionize distilled water.
2. Prepare blank by using blank solution (0 ppm standard) with the same volume of sample solution and adjust to a final volume of 25 ml with de-ionize distilled water.
3. Determine by using an atomic absorption spectrophotometer with absorbance at 766.5 nm.

Analysis of Ca and Mg

1. Dilute the extracted solution to make its concentration into the range of standard Ca and standard Mg (use 0.2-1.0 ml depends on type of plant tissues) to a final volume of 25 ml with La_2O_3 solution.
2. Prepare blank by using blank solution (0 ppm standard) with the same volume of sample solution and adjust to a final volume of 25 ml with La_2O_3 solution.
3. Determine by using atomic absorption spectrophotometer with absorbance at 422.7 nm for Ca, and at 285.2 nm for Mg.

Appendix C-10 Analysis of free amino acid by using HPLC (Ruamrungsri, 1997)

Standard preparation for HPLC analysis

1. Pipette 170 µl standard amino acids solution into a micro-vial.
2. Add 30 µl methanol (without β-AIBA) to a final volume of 200 µl.

Sample preparation for HPLC analysis

1. Pipette extract sample solution (use 0.5-2.5 µl, containing about 100 µM of total amino acid) into a micro-vial.
2. Add 30 µl of β-AIBA methanol (containing 12.5 µM of L-β-Amino-iso-butyric acid, use as the internal standard amino acid).
3. Fill up to 200 µl with 1st buffer solution.

Preparation of reagents

1. β-AIBA methanol

Use 50 µl β-AIBA stock solution (dissolve 182.6 g β-AIBA with 25 ml de-ionize distilled water), fill up to 50 ml with methanol.

2. Buffer solutions

Chemical composition	1st	2nd	3rd	4th	5th	6th
1. tri-lithium citrate-tetrahydrate (g)	14.1	14.1	28.2	42.3	61.1	-
2. methyl alcohol (ml)	30	-	-	-	-	-
3. lithium chloride anhydrous (g)	-	2.12	-	19.1	24.6	-
4. lithium hydroxide (g)	-	-	-	-	-	6.3
5. de-ionize distilled water (ml)	960	980	970	930	930	500
6. β-thiodiglycol (ml)	-	-	2	2	2	-
7. *n*-caprylic acid (µl)	100	100	100	100	100	-
8. perchloric acid (pH adjust, ml)	11	11.5	15	20	15	-
pH ($\pm$0.03)	2.8	3.28	4.25	3.75	4.55	-

3. OPA reagent

Dissolve13.6 g boric acid, 40.7 g sodium carbonate and 18.8 g potassium sulfate in 1,000 ml of de-ionize distilled water, and then add OPA solution (dissolve 0.8 g of o-Phthalaldehyde with 14 ml absolute ethanol by using an ultrasonic generator). Add 1 g N-acetyl-L-cysteine and 2 ml of 30% Briji solution, and then dissolve well. Carefully pour into a bottle to avoid air bubbles. Keep in a refrigerator.

4. Hypo reagent

Dissolve 27.4 g boric acid and 23.0 g potassium hydroxide in a final volume of 1,000 ml with de-ionize distilled water, separate into 2 parts, each of 500 ml and then keep in a refrigerator.

Before using, add 0.2 ml sodium hypochlorite (assay Cl=5%), and then de-aeration with a vacuum pump about 15 minutes.

Preparation of standard amino acid

Standard amino acid type AN	50 µl
Standard amino acid type B	50 µl
2.5 mM L-Asparagine	50 µl
2.5 mM L-Glutamine	50 µl
2.5 mM L-Tryptophan	50 µl
Fill up to 10 ml with 1st buffer solution.	

HPLC system unit

Intelligent HPLC pump	JASCO 880-PU
Solvent selection unit	JASCO 880-03
System controller	JASCO 801-SC
Intelligent sampler	JASCO 855-AS
Intelligent spectrofluorometer	JASCO 820-FP
HPLC pump	JASCO 885-PU

Appendix C-11 Analysis of IAA and ABA by using GCMS

The protocol has been described by Associate Professor Dr. Ohtake Norikuni, Niigata University, Japan (personal communication)

Extraction protocol

1. Weigh 200 mg of homogenize freeze-dried sample, put into a mortar on an ice box.
2. Add 40 ml BHT-Methanol.
3. Add 50 µl 0.501 mM standard ^{13}C-IAA and 50 µl of 0.508 mM ^{2}H-ABA.
4. Gently mill the sample, carefully pour into a centrifuge tube, wash the mortar and pestle twice, each with 10 ml BHT-Methanol.
5. Centrifuge at 8,000 rpm, 4°C for 10 min.
6. Collect the supernatant into a round bottom flask (for vacuum evaporator).
7. Wash the precipitate twice, each with 10 ml of BHT-Methanol and centrifuge at 10,000 and 12,000 rpm, respectively at 4°C for 10 min., collect the supernatant.
8. Evaporate the extract solution in a vacuum evaporator at 40°C until dry.
9. Dissolve the dry extract sample twice with 40 ml and 20 ml diethyl ether, respectively by using an ultrasonic generator, and pour into a separating funnel.
10. Wash again twice with 40 ml and 20 ml of 50 mM phosphate-Na buffer solution (pH8) by using an ultrasonic generator, and pour into the same separating funnel.
11. Shake strongly and continuously at 4°C for 30 min., open the separating funnel valve for 2–3 times to release more vapor pressure during doing in this step.
12. Hold the separating funnel on a separating funnel rack at 4°C in a dark room until the solution is completely separated.
13. Keep the lower portion into a beaker.
14. Repeat twice for step 11 to 13 by using 20 ml of 50 mM phosphate-Na buffer solution (pH 8).
15. Carefully adjust the pH to 2.8 with H_3PO_4 (assay ≥ 85%).
16. Pour into a new separating funnel and wash the beaker twice with 50 ml and 30 ml ethyl acetate, respectively.
17. Strongly and continuously shake at 4°C for 30 min., open the separating funnel valve for 2–3 times to release more vapor pressure during doing in this step.
18. Hold the separating funnel on a separating funnel rack at 4°C in a dark room until the solution is completely separated.
19. Keep the lower portion into a beaker, and carefully pour the upper portion into a round bottom flask.
20. Pour the lower portion into the old separating funnel.
21. Extract the lower portion twice with 60 ml ethyl acetate, follow with step 17 to step 19.
22. Dry the upper portion using a vacuum evaporator at 40°C.

Remark; the dry extract sample can be kept overnight by wrapping the round bottom flask with aluminum foil and keep in a refrigerator.

Purification protocol and determination of IAA and ABA

1. Set up the peristaltic pump to purify IAA and ABA by using Sep-Pak® Plus C18 long body cartridge at flow rate 1 ml/min., connecting to 10 ml syringe.
2. Wash the Sep-Pak® column by loading 10 ml of 100% methanol and follow with 10 ml de-ionize distilled water.
3. Dissolve the dry extract sample (from the step 22 of the extraction protocol) well with 6 ml of 10 % methanol pH 2.8 (adjust with HCl) by using an ultrasonic generator.
4. Wash again twice with 2 ml and 2 ml of 10 % methanol pH 2.8, respectively.
5. Load the sample solution into the syringe by using micro pipette with continuous running.
6. Wash with 10 ml of 10% methanol pH 2.8, pass trough the Sep-Pak® column.
7. Elude IAA and ABA in Sep-Pak® column with 10 ml of 40% methanol and collect into a small test tube, repeat this step again.
8. Dry the purified solution using a vacuum evaporator at 40°C.
9. Dissolve the purified sample well with 100% methanol 200 µl, 100 µl and 100 µl, respectively; use micro pipette to transfer the purified sample solution into a mini-test tube.
10. Dry the purified sample solution using a vacuum evaporator at 40°C.
11. Add 200 µl Diazomethane.
12. Carefully dry with N_2 gas.
13. Dissolve well with 20 µl ethyl acetate
14. Inject 1 µl purified sample solution into GC/3DQMS (use DB5MS column) to determine IAA and ABA contents in the sample by calculating their concentrations from peak areas comparing with standard ^{13}C-IAA and ^{2}H-ABA.

Preparation of reagents

1. BHT-Methanol
   Dissolve 10 mg BHT (Butyl hydroxy toluene) in 100 ml of 80% methanol.

2. 50 mM phosphate-Na buffer solution (pH8)
   Prepare 100 mM Na_2HPO_4 solution (dissolve 7.1 g Na_2HPO_4 in 500 ml de-ionize distilled water), and also prepare 100 mM NaH_2PO_4 solution (dissolve 6.0 g NaH_2PO_4 in 500 ml de-ionize distilled water).
   Pour 100 mM Na_2HPO_4 solution into 1L beaker and continuously stir on a magnetic stirrer plate. Carefully pour 100 mM NaH_2PO_4 solution into the first solution until the buffer solution has pH = 8.0
   Dilute the 100 mM phosphate-Na buffer solution (pH8) into 50 mM by using an equally volume of buffer solution and de-ionize distilled water.

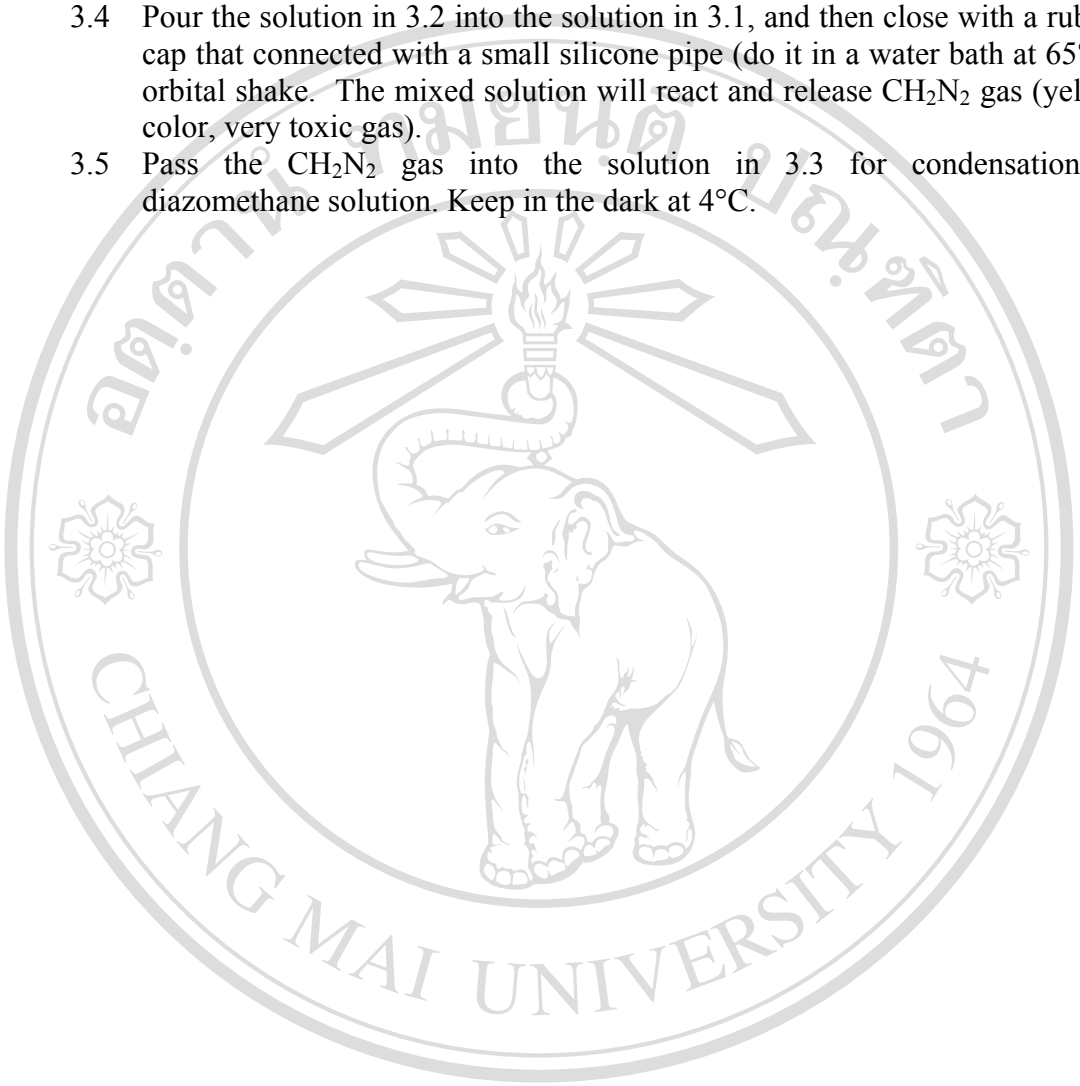
3. Diazomethane (CH_2N_2)
   3.1 Weigh 4.3 g N-methyl-N-nitroso-p-toluene sulfonamide, put into 250 ml flask, and then dissolve with 25 ml diethyl ether (do well in an ice box).

3.2 Dissolve 1.2 g KOH with 2 ml de-ionize distilled water and then add 4 ml absolute ethanol, keep it cool in an ice box.

3.3 Prepare 20 ml diethyl ether in 50 ml flask and keep it cool in an ice box.

3.4 Pour the solution in 3.2 into the solution in 3.1, and then close with a rubber cap that connected with a small silicone pipe (do it in a water bath at 65°C), orbital shake. The mixed solution will react and release CH_2N_2 gas (yellow color, very toxic gas).

3.5 Pass the CH_2N_2 gas into the solution in 3.3 for condensation to diazomethane solution. Keep in the dark at 4°C.



Curriculum vitae

Name Theeraphon Phornsawatchai

Date of birth July 13, 1966

Education

Certificate	Institute	Academic year
Mathayom 5	King's College, Nakornpathom	1982
B.Sc. (Biology)	Faculty of Science, Srinakharinwirot University, Songkhla	1986
M.S. (Horticulture)	Faculty of Agriculture, Chiang Mai University, Chiang Mai	1992

E-mail t.phorns@chiangmai.ac.th

Research experience

1990-1997 Researcher
H.M. The King's Initiative Centre for Flower and Fruit Propagation
c/o Faculty of Agriculture, Chiang Mai University, Chiang Mai

1997- Researcher
Institute for Science and Technology Research and Development
Chiang Mai University, Chiang Mai