APPENDIX

Appendix A Plant microtecnique (Johansen, 1940)

Reagent preparation

Fixation

FAA (Formalin, Acetic acid, and Alcohol) fixative contains:

- EtOH 95% 50 ml
- Acetic acid 5 ml
- Formalin (37% Formaldehyde) 10 ml
- H_2O 35 ml

Dehydration series

Incubate sample for 1 day, depending on toughness and size, in:

- EtOH 70%
- EtOH 85%
- EtOH 95%
- EtOH 100%

Stain

Hematoxylin contains:

- Hematoxylin 6 g

- Sodium iodate 0.6 g
- Aluminum sulfate 52.8 g
- Distilled water 690 ml
- Ethylene glycol 250 ml
- Glacial acetic acid 60 ml

Analysis methods

Determination of the developmental stage of the inflorescence formation was done by using the paraffin embedded technique and a method described by Johansen (1940). Five shoot and inflorescence were sampling at 0-5, 6-10, 11-15, 16-20 and 21-25 cm. The shoot samples were stopped activity and fixed with fixative reagent (FAA) about 1 week. Then they were suctioned by vacuum pump at 6,000 mm Hg for 1 hrs and left under vacuum condition for at least 24 hrs until bubble was not occurred. After that shoot and inflorescence samples were dehydrated for 24 hrs in a solution containing of tertiary butyl alcohol and mixed with 5 series of 50, 70, 85, 95 and 100% alcohol respectively. tertiary butyl alcohol plus alcohol 100% erythosine was added as dye. Ease samples were then infiltrated 3 times with pure tertiary butyl alcohol for 12 hrs each then transferred into solution of pure tertiary butyl alcohol mixed with paraffin oil (1:1), and pure paraffin oil for each 12 hrs respectively. After that the samples were kept in paraplast solution in the oven (55–60 ^{o}C) abount 2-3 months and embedded in paraplast. Thus, the paraplast samples were sectioned at a thickness of 10 µm using rotary microtome, and affixed on the slide with 2% Hapt's adhesive. The slides of microtome section were studied and photographed under stereo

microscopy.

Appendix B Total non-structural carbohydrate analysis (TNC) by Nelson method (Smith, 1964).

Sample extraction

1. Weight 0.20 g sample, add 40 ml of 0.2 N H_2SO_4 and cover flask with aluminum foil.

2. Heat at 100^oC for 1 hrs, after keep flask to cooling at room temperature.

3. Adjust pH the solution about 7.00, add deionized water to 100 ml.

4. Take solution to filter with Whatman No 5 or 42.

5. Keep the solution in plastic bottle for analysis.

Reagent preparation

1. Nelson's alkaline copper reagent

Prepare solution No.1 by dissolve 25 g of anhydrous sodium carbonate (Na_2CO_3) into 250 ml deionized water, after added 12 g of potassium sodium tartrate ($C_4H_4KNO_6.4H_2O$) and 40 ml of 10% copper sulfate (Make 4 g $CuSO_4.5H_2O$ dissolve into deionized water to 40 ml), add 16 g of sodium bicarbonate. The Solution No.2 by dissolve make 180 g of anhydrous sodium sulfate (Na_2SO_4) into 500 ml of deionized water. Mix solutions No.1 with No.2 adjust to 1 L, after about 1 week, take to filter and keep at 30 – 37 ^oC.

2. Arsenomolybdic acid reagent

Prepare solution No.3 by dissolve 50 g of ammonium molybdate ($(NH_4)_6AsO_{24}.4H_2O$) into 90 ml of deionized water and add 42 ml H_2SO_4. Solution No.4 prepared by dissolve 6 g of disodium hydrogen arsenate ($Na_2HAsO_4.7H_2O$) into 50 ml of deionized water. Then mix solution No.3 with No.4 and adjust to 1 L, keep at 30 – 37 ^oC.

Analysis method

1. Standard solution was made by dissolving D-glucose. Make standard solution concentration 0 - 1 ppm.

2. Add 1 ml of Nelson's alkaline copper reagent, mix well and cover flask with aluminum foil.

3. After take the flask in water bath at 100 ^oC for 20 min. keep flask to cooling at room temperature.

4. Add 1 ml of arsenomolybdic acid reagent, shaking vigorously and add deionized water to 25 ml with mix well, keep at room temperature for 30 min.

5. Determine the absorbance at 540 nm.

6. Calculation;

$$TNC = \frac{\text{(mg glucose equivalent) x (mg D-glucose/ mg dry weight)}}{\text{Weight of sample}}$$

Appendix C Total nitrogen analysis by modified Kjeldahl method (Ohyama *et al*., 1985).

Reagent

- Reagent A (EDTA reagent);

Dissolve 6 g of EDTA (ethylenediaminetetra acetic acid disodium salt) into80 ml of deionized water, adjust pH about 7, mix well and dilute to a final volume of 100 ml.

- Reagent B (1 M of KH_2PO_4);

Dissolve 136.09 g KH_2PO_4 and 2.75 g benzoic acid into 1 L of deionized water.

-Reagent C (Phenol-nitroprusside reagent);

Dissolve 100 mg sodium nitroprusside into 10.25 ml phenol, dilute to a final volume of 1 L with deionized water (Use the sodium nitroprusside as a catalyst).

- Reagent D (Buffer hypochlorite reagent);

Put 10 g NaOH (adjusts pH 10 by 10 N of NaOH), 7.06 g Na_2HPO_4 and 31.8 g $Na_3PO_4.12H_2O$ into a 500 ml beaker, dissolve in deionized water and transfer to 1 L of volumetric flask, add 10 ml of sodium hyperchlorite, dilue to 1 L of flask with deionized water.

-Standard ammonium solution;

Dissolve 471.7 mg $(NH_4)_2SO_4$ in 1 L of 0.5 N H_2SO_4 for 100 mg/l of a stock solution. Make standard concentration 0 -0.7 mg/l.

Analytical method

- Pipette sample solution of the H_2SO_4 digested solution 0.1 - 2 ml into a 25 ml of volumertric flask, add 0.5 ml of reagent A and 0.5 ml of reagent B.

-Add a small amount of 2 N NaOH, for pH adjust, until color changed, add 2.5 ml of reagent C, follow by 2.5 ml of reagent D, and then fill up flask to volume with deionized water and mix well.

-Maintain the flask at 30 ^{o}C for 3 hrs and determine the absorbance of the colored complex at a wavelength of 625 nm. Do the same method for blank solution and standard.

-Determine the NH_{4+}-N concentration of the sample by reference to a calibration curve plotted form the results obtained with a standard curve.

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