CHAPTER 3

MATERIALS AND METHODS

1. MATERIALS

1.1 Plant materials

1.1.1 Experiment 1: Effects of defoliation and girdling on panicle position of potassium chlorate treated trees.

In this experiment one year old grafted longans cv. Daw were used. The plants were grown in pots, 15.24 centimeters (6 inches) in diameter with soil base media (soil:compost:sand = 3:1:1) in the fruit tree nursery of the Department of Horticulture, Chiang Mai University (latitude 18°47 N, 312 m MSL), Chiang Mai, Thailand.

1.1.2 Experiment 2: Effects of leaves maturity on some isozymes changes in potassium chlorate treated longan trees

Three years old grafted longans cv. Daw were used in both experiments. They were grown in pots, 38.10 centimeters (15 inches) in diameter with soil base media (soil:compost:sand = 3:1:1) in the same nursery as in the Experiment 1.

1.1.3 Experiment 3: Effects of potassium chlorate on changes of proteins in longan leaves during flowering period.

Plant materials were same as in the Experiment 2.

1.2 Scientific apparatus

1.2.1 Analytical balance, Model 211S, Sartorious, Germany.

1.2.2 Spectrophotometer, HITACHI model U-2001, Japan.

1.2.3 Centrifuge, Universal Bench Top, Hetich, Germany.

1.2.4 Electrophoresis cell apparatus, Bio-Rad model Mini protein 3, England.

1.2.5 Power supply for electrophoresis, Amersham model EPS 601, Sweden.

1.2.6 Block heater, Wealtec Model HB-2, USA.

1.2.7 Electrophoresis cell apparatus, Hoefer model SE 600, Sweden.

1.2.8 Power supply for electrophoresis, Amersham model SE800, England.

1.2.9 Electro-Eluter, Bio-Rad model 422, England.

1.2.10 Orbital Shaker, ArmaLab Model OR 100-20, USA.

1.2.11 Tran-Blot® SD Semi-Dry Electrophoretic Transfer Cell, BIO-RAD, England.

1.2.12 Procis™ cLC protein sequencer, Procis, USA.

1.2.13 Freezer, Refrigerator (-20 °C) Model SF-C992, Sanyo, Japan.

1.3 Chemical reagents

1.3.1 Reagents for isozymes electrophoresis.

1.3.1.1 Extraction buffer

1.3.1.1.1 0. 2 M. Tris-HCl buffer pH 8.2

1.3.1.2 Gel

1.3.1.2.1 acrylamide/bis (30% T, 2.67% C)

1.3.1.2.2 3 M. Tris-HCl buffer pH 8.8 (for separating gel)

1.3.1.2.3 0.5 M. Tris-HCl buffer pH 6.7 (for spacer gel)

1.3.1.2.4 Ammonium persulphate

1.3.1.2.5 5x Electrode buffer

1.3.1.2.6 0.5% Dye marker

1.3.1.3 Enzyme staining and incubation.

1.3.1.3.1 Peroxidase (modified by Soltis *et al* , 1983)

1.3.1.3.2 Esterase (Brewer, 1970)

1.3.1.3.3 Shikimic dehydrogenase (Soltis *et al*, 1983)

1.3.1.3.4 Malate dehydrogenase (Harris and Hopkinson, 1978)

1.3.1.3.5 glucose-6-phosphate dehydrogenase (Brewer, 1970)

1.3.1.3.5 Supperoxidase dismutase (Healy and Mulcahy, 1979)

1.3.1.4 Stain-fixing solution

1.3.1.4.1 1:5:5 Glacial acetic: methanol: water

1.3.1.4.2 50% glycerol (in water)

1.3.2 Protein assay

1.3.2.1 0.1 M phosphate buffer saline pH 6.0

1.3.2.2 Bradford reagent (Bradford, 1976)

1.3.3 SDS-PAGE

1.3.3.1 acrylamide/bis (30% T, 2.67% C)

1.3.3.2 1.5 M Tris-HCl, pH 8.8

1.3.3.3 0.5 M Tris-HCl, pH 6.8

1.3.3.4 10% (w/v) SDS

1.3.3.5 10 % Ammonium persulfate (w/v)

1.3.3.6 Sample buffer

1.3.4 Elution

1.3.4.1 Elution buffer

1.3.5 Semi-dry Blotting

1.3.5.1 Towbin transfer buffer

2. METHODS

2.1 Experiment 1: Effects of defoliation and girdling on panicle position of potassium chlorate treated trees.

One year old grafted longan plants were grown until the leaves were mature. The experiment was started by defoliation and/or girdling, at various positions. Two grams of potassium chlorate were applied to the plants except those of the control treatments by soil drenching. The treatments are shown in Table 1.

Table 1 Treatments of girdling and defoliation of potassium chlorate treated longan trees.

Treatment	Girdling site	Defoliation site	Treated with potassium chlorate
1	None	None	No
2	None	None	Yes
3	None	1st site	Yes
4	None	2nd site	Yes
5	None	1st site and 2nd site	Yes
6	1st site	None	Yes
7	1st site	1st site	Yes
8	1st site and 2nd site	None	Yes
9	1st site and 2nd site	1st site	Yes
10	1st site and 2nd site	2nd site	Yes
11	1st site and 2nd site	1st site and 2nd site	Yes
12	1st site	2nd site	Yes
13	1st site	1st site and 2nd site	Yes

Site of girdling: girdlings were done at two different positions on the shoots i.e. at the 2nd and 4th internodes (1st site and 2nd site, respectively) from the terminal bud which are the stems (internodes) between 2nd and 3rd compound leaves (1st site) and internodes between 4th and 5th compound leaves (2nd site) (Figure 3). During experimental time, wounding phloem developed in girdling zone from the vascular cambium was discarded.

Site of defoliation: defoliation was also done at two position, i.e. at the first and second compound leaves (1st site) and at the third and forth compound leaves (2nd site)(Figure 4).

All treated and untreated plants were grown free from disease and insects until flowering (18-21 days after treatment)

The experimental design used in this experiment was completely randomized design (CRD). Each treatment composed of 3 replications. Records were done as follows:-

1) Flowering percentage of each treatment.

2) Panicle position of each branch was defined as
    a. Terminal bud refers to the bud at the apex or terminal shoot.
    b. Second axillary bud or buds refer to the bud/buds which located between 1st and 2nd site of girdling.
    c. Third axillary buds refer to the bud or buds that located under 2nd site of girdling.

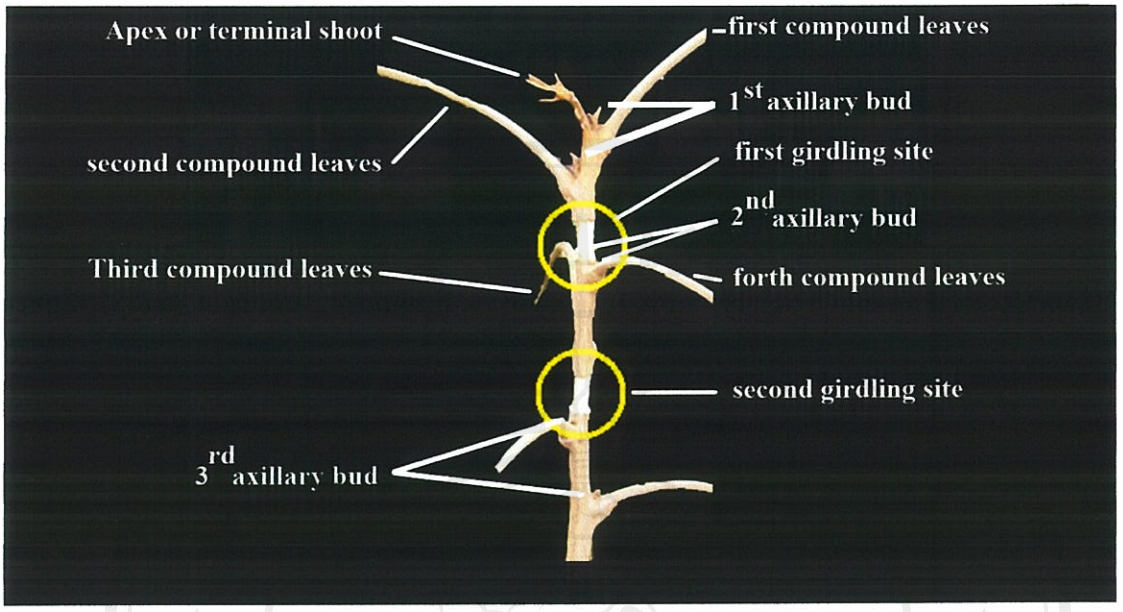

Figure 3 The positions girdling sites, apex or terminal shoot, compound leaves and axillary buds on a longan branch.

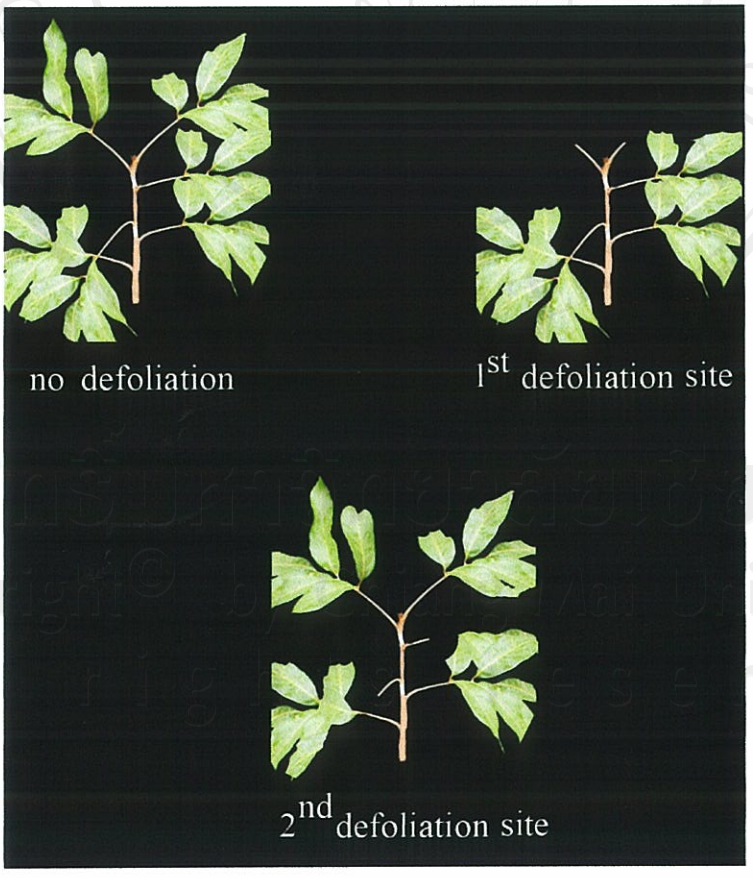

Figure 4 Defoliation sites of longan shoots.

2.2 Experiments 2: Effects of leaves maturity on some isozymes changes in potassium chlorate treated longan trees.

This experiment was carried out with the three years old grafted longan. Complete defoliation was done with the shoots of all plant from the tips to 40 centimeters downwards. Leaves of different stages of maturation after leaf flushing were collected for protein content analysis. There were 6 treatments in this experiment:

Treatment 1: leaves of 15 days after flushing, without potassium chlorate treatment.

Treatment 2: leaves of 15 days after flushing, treated with 2 grams potassium chlorate.

Treatment 3: leaves of 30 days after flushing, without potassium chlorate treatment.

Treatment 4: leaves of 30 days after flushing, treated with 2 grams potassium chlorate.

Treatment 5: leaves of 45 days after flushing, without potassium chlorate treatment.

Treatment 6: leaves of 45 days after flushing, treated with 2 grams potassium chlorate.

The leaflets for protein and isozymes analyses of each treatment were collected from the 2nd and 3rd leaflets of the 2nd and 3rd compound leaves at 0, 1, 3, 5, 7, 14, 21 and 28 days after treatment. The leaflets were rinsed immediately with mild detergent solution followed by twice of distilled water (Domoto, 2005) and then the samples were put in an icebox during transporting to the laboratory. The samples were stored at -20 ^{o}C until used for protein and isozymes analyses.

2.2.1 Preparing of isozyme samples (Modified from Hiratsuka, 1992)

1) Grind the longan leaflets in a mortar with liquid nitrogen. Add 3 ml of isozymes extraction buffer (see appendix) per gram of tissue, 2% of polyvinylpoyrrolidone (PVPP) and grind the sample.

2) Filter through muslin and centrifuge at RCF 1,033.04 X G, 4°C for 30 min in refrigerated centrifuge transfer the supernatants to new 1.5 ml micro centrifuge tubes and add 10 % of glycerol (about 150 µl per 1.5 ml of sample).

3) Store isozyme samples at -20°C until ready for isolating by electrophoresis.

2.2.2 Gel electrophoresis of isozymes (Modified from Ornstein, 1964; Davis, 1964)

1.) Clean the internal surfaces of the gel glass plate of Mini protein III (Figure 5) with ethanol, dry, and then join the gel glass plate together using 1.5 millimeter spacers. The gel casting stand is then assembled. The black cams are turned to seal the bottom surface of the sandwich into the casting stand gasket.

2) Mix the 7.0 % separating gel solution by adding, 3 ml of acrylamide/bis (30% T, 2.67% C), 3.125 ml of 1.5 M Tris-HCI pH 8.8 (resolving gel buffer), 6.25 ml of deionized water, 125 µl of ammonium persulphate (fresh) and 15 µl of N,N,N',N'-Tetramethylethylenediamine (TEMED) (add TEMED after deaeration). Gently swirl the flask to mix.

3) Use a 0.1 milliliter Pasteur pipette, transfer the separating gel solution to the center of the sandwich along an edge of one of the spacers until the height of solution in the sandwich 4 centimeter from the top.

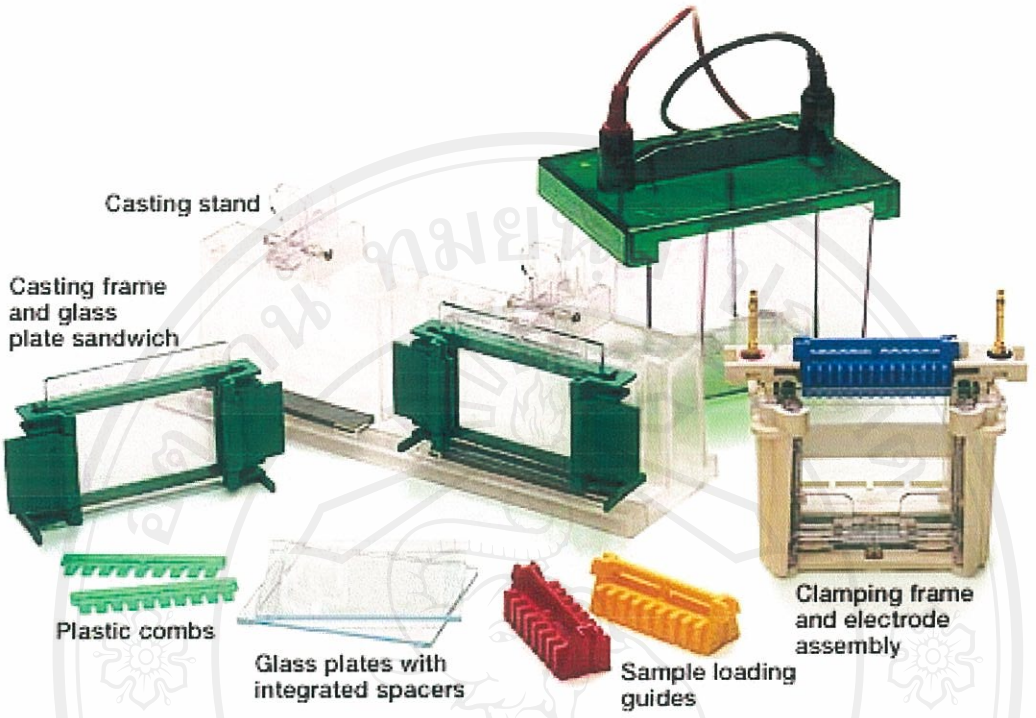

Figure 5 Components of a BioRAD Mini-Protein 3 gel electrophoresis unit.

4) Fill a transfer pipette with water-saturated n-butanol. Position the pipette or needle at about a 45° angle with the point at the top of the acrylamide next to a spacer. Gently apply approximately 0.3 ml of n-butanol. Repeat on the other side of the slab next to the other spacer. The n-butanol will layer evenly across the entire surface after a minute or two. Repeat this process to overlay the second slab. A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10-20 minute. The gel should be fully polymerized in about 30-40 min.

5) After polymerization, tilt the casting stand to pour off the overlay and rinse the surfaces of the gels twice with resolving gel buffer overlay.

6) Mix the 4.0% stacking gel solution by adding, 1.3 ml of acrylamide/bis (30% T, 2.67% C), 2.5 of 0.5 M Tris-HCI pH 6.8 (resolving gel buffer), 6.0 ml of deionized water, 50 µl of ammonium persulphate (fresh) and 10 µl of TEMED (add TEMED after deaeration). Gently swirl the flask to mix.

7) Poured off the resolving gel buffer overlay from the gel. Remove all liquid before proceeding.

8) Fill each sandwich with stacking gel solution and insert a comb into each sandwich, taking care not to trap any bubbles below the teeth of the comb.

9) Allow the gel to sit for at least 30 min. A very sharp interface would be visible when the gel has polymerized. This should be visible within 10-20 minutes. The gel should be fully polymerized after 30 minutes and after gel polymerized take off the comb.

10) Take the glass plate sandwich from frame and cast in clamping frame and electrode assembly. Bring electrode assembly in to buffer tank.

11) Dilute 5 x Electrode buffer with deionized water 5 times and fill diluted electrode buffer in buffer tank until the level to upper level and fill diluted buffer in electrode assembly until diluted buffer to upper of glass plate sandwiches. Cover buffer tank with tank lid and take it in to ice box. Connected the power supply to the anode and cathode of the gel apparatus and pre-run at 20 mA (constant currents) for 15 minutes.

12) Mix the isozyme samples with dye marker by samples: dye marker ratio 4:1 and loading mixed isozyme samples in each well as show in Table 2

13) Adjust the power supply to 25 mA and run electrophoresis until the dry marker reach the end of glass plate. Take the glass plate sandwiches from electrode assembly. Strip the glass off from gel and rinse the gel with distilled water twice.

Table 2 Isozymes samples loading in each well number.

Well number	Isozyme sample marking (days after treatment)	Leaflet age (days after flushing)
1	0	15
2	1	16
3	3	19
4	5	24
5	7	31
6	14	38
7	21	45
8	28	52

14) Take the gel into an incubation box and stain isozymes in the gel with specific enzymes, peroxidase, esterase, shikimic dehydrogenase, malate dehydrogenase, supperoxidase dismutase and glucose 6 phosphate dehydrogenase staining reagents (see appendix 1.3).

15) The stained gel is washed in distilled water twice and immersed in 7 % acetic acid to destain and fix isozymes in the gel.

16) Take photos of the gel and analyze zymogram patterns of the isozymes.

2.3 Experiment 3: Effects of potassium chlorate on changes of proteins in longan leaves during flowering period.

This experiment used 48 of three years old grafted longan plants. They were divided into two groups. The first group was treated with potassium chlorate 10 g per pot and another group was untreated. The leaf samples were collected as described in experiment 2.

2.3.1 Experiment 3.1: Effect of leaf age on protein contents in longan leaves during flowering period.

This experiment was carried out with the three years old grafted longan. Complete defoliation was done with the shoots of all plant from the tips to 40 centimeters downwards. Leaves of different stages of maturation after leaf flushing were collected for protein content analysis. There were 6 treatments in this experiment:

Treatment 1: leaves of 15 days after flushing, without potassium chlorate treatment.

Treatment 2: leaves of 15 days after flushing, treated with 2 grams potassium chlorate.

Treatment 3: leaves of 30 days after flushing, without potassium chlorate treatment.

Treatment 4: leaves of 30 days after flushing, treated with 2 grams potassium chlorate.

Treatment 5: leaves of 45 days after flushing, without potassium chlorate treatment.

Treatment 6: leaves of 45 days after flushing, treated with 2 grams potassium chlorate.

The leaflets for protein analysis of each treatment were collected from the 2nd and 3rd leaflets of the 2nd and 3rd compound leaves at 0, 1, 3, 5, 7, 14, 21 and 28 days after treatment. The leaflets were rinsed immediately with mild detergent

solution followed by twice of distilled water (Domoto, 2005) and then the samples were put in an icebox during transporting to the laboratory.

2.3.1.1 Protein contents assay

Determination of protein contents of sample was conducted using Bradford Assay technique (Bradford, 1976) as follow:

1) Preparation standard protein: Dissolve 0.25 g of bovine serum albumin (BSA) in 25 ml of distilled water. Prepared solution concentration was 10 mg/ml. Dilute 0.5 ml of 10 mg/ml standard protein with 0.1 M phosphate buffer saline pH 6.0 (0.1 M PBS pH 6.0) to 25 ml. Finished solution concentration was 200 µl/ml.

2) Pipette 0, 50, 100, 150, and 200 µl of 200 µl/ml standard protein into 5 test tubes and dilute to 0.1 M. PBS pH 6.0 to 300 µl. The known sample protein concentrations were 0, 0.01, 0.02, 0.03 and 0.04 mg/ml respectively.

3) Add 3 ml of Bradford reagent into each tube of known sample proteins and incubated the sample for 10 minutes.

4) Record the absorbance of the known sample at 595 nm.

5) Prepare a standard curve of protein contents (Y-axis) and absorbance value (X-axis) and calculate the linear regression equation ($y = 18.1x + 0.016$) and coefficient of regression ($R^2 = 0.9655$) (Figure 6).

6) Determinate the amount of protein in leaflet samples (crude protein) by using 200 µl of extracted crude protein (obtained by centrifuging the leaf juices at relative centrifugal force (RCF) of 1,033.04 X G, 4°C for 15 min). Add 100 µl 0.1 M PBS pH 6.0 and 3 ml of Bradford reagent. Vertex to homogenize and recorded the absorbance of the samples at 595 nanometer. Absorbance values are calculated with a regression equation from the standard curve and converted as the protein content (mg/ml).

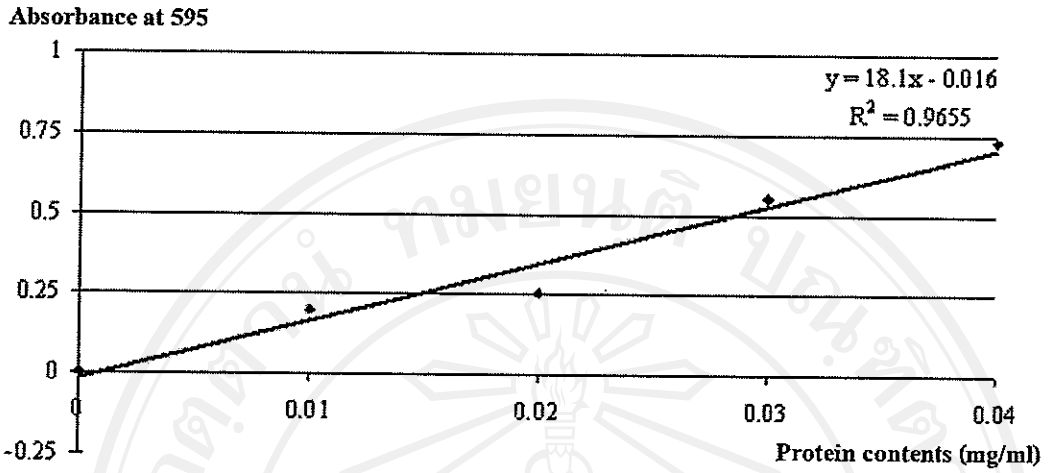

Figure 6 Standard curve of protein (mg/ml) for protein contents analysis in Experiment 3.1: Effect of leaf age on protein contents in longan leaves during flowering period.

2.3.2 Experiment 3.2: Effects of potassium chlorate on changes of proteins in longan mature leaves during flowering period.

2.3.2.1 Protein extraction from leaves

Protein extraction method was modified from Nelson *et al.* (2002). The extraction of protein in longan leaves as described in Experiment 2 except used grinded samples add 3 ml of protein extraction buffer. Filter the crude protein through muslin and centrifuge at RCF 1,033.04 X G at 4°C for 30 minutes. The supernatants were taken to new 1.5 ml micro centrifuge tube and add 10 % of glycerol and store at -20°C until analysis by SDS-PAGE on item 2.3.2.3

2.3.2.2 Calculate volume of sample use

Determination the amount of protein present in sample by Bradford Assay (Bradford, 1976) that method as described in Experiments 3.1 Prepare a standard curve between protein contents (Y-axis) and absorbance value (X-axis) and calculation the linear regression equation ($y = 18.183x + 0.0448$) and coefficient of regression ($R^2 = 0.9774$) (Figure 7). Determinate the amount of proteins present in unknown sample (crude protein) as described in Experiments 3.1 and

calculates the amount of protein for loading. Determinate amount of protein contents in each sample is 20 µg.

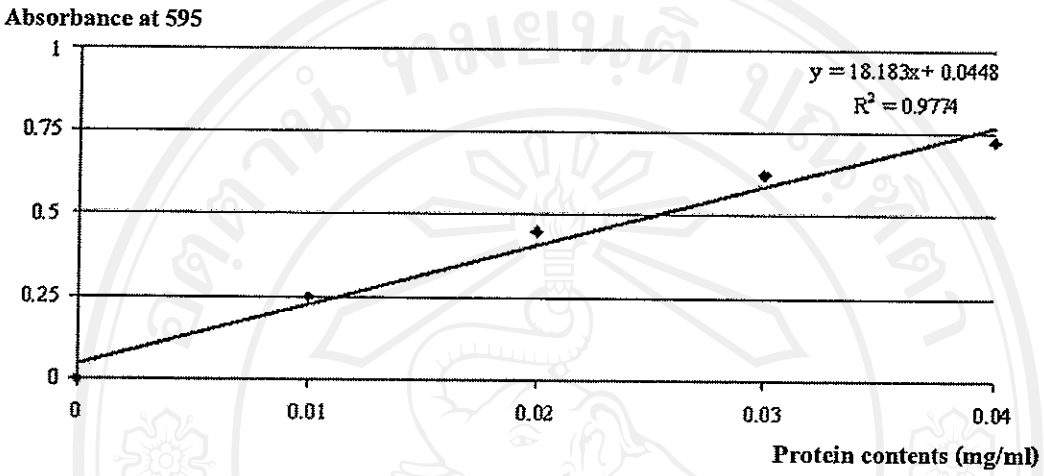

Figure 7 Standard curve of protein (mg/ml) for protein contents analysis in Experiment 3.2: Effects of potassium chlorate on changes of proteins in longan leaves during flowering period

2.3.2.3 Prepare of protein samples (Modified from Hiratsuka, 1992)

1) Grind the longan leaflets in a mortar (stored in -20°C before use) with liquid nitrogen. Add 3 ml of protein extraction buffers (see appendix) per gram of tissue, 2% of polyvinylpoyrrolidone (PVPP) and grind the sample.

2) Filter through muslin and centrifuge at RCF 1,033.04 X G, 4°C for 30 min in refrigerated centrifuge transfer the supernatants to new 1.5 ml micro centrifuge tubes and add 10 % of glycerol (about 150 µl per 1.5 ml of sample).

3) Store protein extracts samples in -20°C until ready for isolating by electrophoresis.

2.3.2.3 Prepare the separating gel and stacking gel

1.) Clean the internal surfaces of the gel glass plate of Hoffer SE 600 Ruby (Figure 8) with ethanol, dry, and then join the gel glass plate together using 1.5 mm spacers. The gel cast stand is than assembled. The black cams are turned to seal the bottom surface of the sandwich into the casting stand gasket.

2) Mix the 12.5% separating gel solution by adding, 33 ml of acrylamide/bis (30% T, 2.67% C), 20 ml of 1.5 M Tris-HCI pH 8.6 (resolving gel buffer), 25.5 ml of deionized water, 0.8 ml of 10% SDS, 400 µl of ammonium persulphate (fresh) and 20 µl of TEMED (add TEMED after deaeration). Gently swirl the flask to mix.

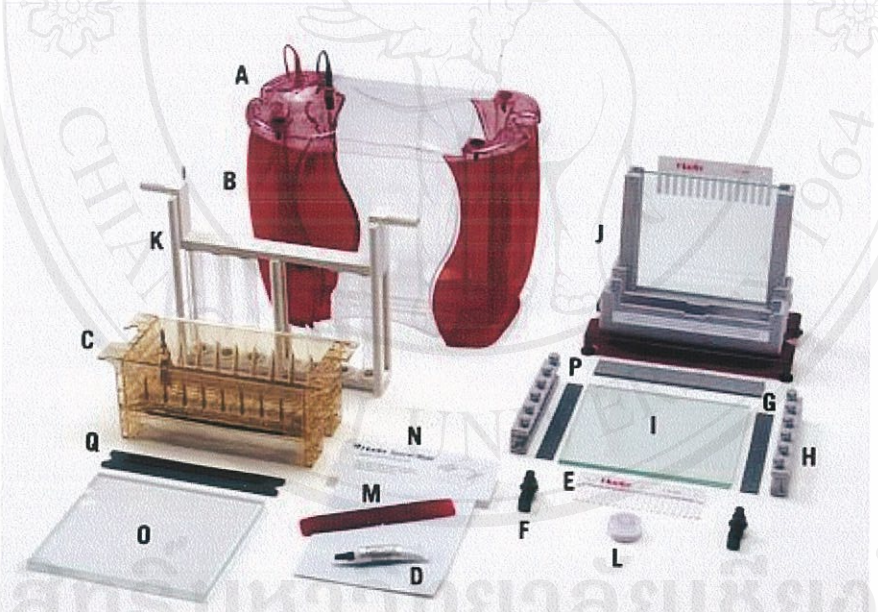

Figure 8 SE 600 Ruby vertical slab gel unit and accessory. A. lid assembly for SE 600 Ruby B. lower buffer chamber for SE 600 Ruby™, C. upper buffer chamber, D. gel seal, E. comb, F. cams, G. spacer, H. clamp 16 cm, I. glass plate 18 × 16 cm, J. casting stand, K. heat exchanger, L. level, M. wonder wedge separator, N. spacer-mate, O. buffer dam, P. laminated rubber gasket, Q. slotted rubber gasket,

3) Using a 25 ml pasteur pipette, transfer the separating gel solution to the center of the sandwich along an edge of one of the spacers until the height of solution in the sandwich 4 centimeters from the top.

4) Fill a transfer pipette with water-saturated n-butanol. Position the pipette or needle at about a 45° angle with the point at the top of the acrylamide next to a spacer. Gently apply approximately 0.3 ml of n-butanol. Repeat on the other side of the slab next to the other spacer. The n-butanol will layer evenly across the entire surface after a minute or two. Repeat this process to overlay the second slab. A very sharp liquid-gel interface was visibled when the gel had polymerized. This should be visible within 10-20 minutes. The gel should be fully polymerized in about 30-40 minutes.

5) After polymerization, tilt the casting stand to pour off the overlay and rinsed the surfaces of the gels twice with resolving gel buffer overlay.

6) Mix the 4.0% stacking gel solution by adding, 2.66 ml of acrylamide/bis (30% T, 2.67% C), 5.0 ml of 1.5 M Tris-HCI pH 8.6 (resolving gel buffer), 12.0 ml of deionized water, 0.2 ml of 10% SDS, 100 µl of ammonium persulphate (fresh) and 10 µl of TEMED (add TEMED after deaeration). Gently swirl the flask to mix.

7) Pour off resolving gel buffer overlay from the gel. Remove all liquid before proceeding.

8) Fill each glass plate sandwich with stacking gel solution and inserted a comb into each sandwich, taking cared not to trap any bubbles below the teeth of the comb.

9) Allow the gel to sit for at least 30 minutes. A very sharp interface was seen when the gel had polymerized. This should be visible within 10-20 minutes. The gel should be fully polymerized after 30 minute and after gel polymerized take off the comb.

10) Fill the lower buffer chamber with 4 liter of 1X SDS/electrode buffer. Installed the sealing gaskets on the upper buffer chamber and put it in place on the gel sandwiches. Remove the lower clamp. Put the upper buffer chamber in place on the heat exchanger in the lower buffer chamber. Carefully fill the upper buffer chamber with tank buffer. Put the lid on and connected the power supply to the anode and cathode of the gel apparatus and pre-running at 30 mA (constant currents) for 15 minutes.

2.3.2.4 Denaturing of protein sample.

1) Take the protein sample of 20 µl and combine equal volumes to 2X SDS/sample buffer in micro centrifuge tube and heat in heat block at 90°C for 5 minutes.

2) Place samples briefly on ice until used.

2.3.2.5 Sample loading.

1) After pre-run, turn off the power supply and take the lid off.

2) Use a 100 µl Hamilton syringe loads the protein ladder and protein samples in each well carefully applying the ladder/samples as thin layer at the bottle of the wells. The volume of protein sample is follow as Table 3.

Table 3 The volume of protein samples in each day which contained 20 µg of crude proteins.

Treatments	Volume of protein sample (µl)							
	Days after treatment							
	0	1	3	5	7	14	21	28
Treated chlorate	16.0	14.8	13.8	14.8	14.2	10.6	10.2	9.0
Non-treated chlorate	16.6	15.4	16.6	14.8	15.4	13.8	14.2	14.8

2.3.2.6 Running the gel and disassemble the gel

1) Connected the power supply to the anode and cathode of the gel apparatus and running at 60 mA. The process of electrophoresis was started, the voltage should be 70-80 V and increase during the run. Kept a record of the voltage and current readings so that future runs could be compared and current leaks or incorrectly made buffers could be detected.

2) When the tracking dye reaches the bottom of the gel, turn the power supply off and disconnect the power cables. Remove the buffer and disassembled the sandwiches by gently loosening and sliding away both spacers. Slip an extra spacer into the bottom edge and separated the plates. Carefully lift the gel into a tray of staining solution.

2.3.2.7 Staining gels with coomassie brilliant blue

1) Place the gel into a tray of coomassie brilliant blue R-250 staining solution.

2) Agitate slowly for 3 hours on orbital shaker.

3) Pour off the staining solution, it could be reuse, and rinsed gel with deionized water 2 or 3 times until the gel was clean. Cover the gels with destain solution agitate slowly for 1-2 hours on orbital shaker.

4) The destain solutions are discard and replace with fresh solution. Repeat until the gel is clear except for the protein bands.

5) Took the stained gel to gel document for taking a photograph and analyzed bands of protein.

2.3.2.8 Electro elution of protein from SDS-PAGE

After the protein samples had been separated by SDS-PAGE, the protein bands of interest must be localized for excision and elution. This process had 2 steps.

2.3.2.8.1 The first step: preparing of electro-eluter unit.

1) Soak the membrane caps in protein elution buffer for at least 1 h at 60°C membrane caps could be soaking for longer than 1 hour. Glove was worn when handling the membrane caps to prevent the dialysis membranes from becoming contaminate. Membrane caps could be reused for at least five complete runs without a decrease in protein yield. In this case, they were being stored in elution buffer containing 0.05% sodium azide at 4°C.

2) Take the number of glass tubes required for the gel slices to be eluted and placed one frit in the bottom of each tube (Figure 9). Inserted the glass tube and frit into the electro-eluter module and fill any empty grommet holes with stoppers.

3) Place one pre-wetted membrane cap in the bottom of each silicone adaptor. Fill the adaptor with elution buffer and pipette the buffer up and down to remove any air bubbles around the dialysis membrane.

4) Slice the silicone adaptor with the membrane cap onto the bottom of the glass tube with frit. Pull the silicone adaptor partially on and off a few times to ensure that all the air bubbles were expelled.

5) Fill each tube with elution buffer and placed the gel slice into the tube. To increase sample recovery, several bands may be excised from the gel, pooled together, and minced, so long as the height of the gel within the glass tube was approx 1 cm or less. If the glass tube was filled higher than 1 cm, then the time required for elution was increased.

6) Place the entire module into the buffer chamber. Filled the lower buffer chamber with 600 ml elution buffer or the level of the lower buffer was above the top of the silicone adaptors or bubbles might form on the bottom of the dialysis membrane. Fill the upper buffer chamber with 100 ml of elution buffer.

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14) Using a new plastic pipette, removed the remaining liquid in the membrane cap into a microcentrifuge tube. The volume should be around 400 μL. Rinse the membrane cap with 200 μL fresh elution buffer and add to the sample in the microcentrifuge tube. This solution contains the eluted protein.

15) Repeat steps 12 – 14 for each glass tube.

2.3.2.8.2 The Second step: protein precipitation after electroelution.

After electro-elution, the proteins enrich solution also contain SDS, Coomassie brilliant blue R-250 stain, buffer salts, and other contaminants. These can be readily removed by precipitation of the protein before being redissolve for subsequent analysis.

1) Lyophilize the electro-eluted protein sample or dry using a rotary vacuum evaporator.

2) Add 50 μL of water.

3) Add 450 μL of ice-cold acetone acidified to a final concentration of 1 mM HCl and incubate for 3 hours at -20°C.

4) Pellet the precipitated protein using a microcentrifuge.

5) Wash the pellet three times with 100 μL ice-cold acetone and dry the pellet in air. Dissolve the pellet with solvent.

2.3.2.9 Blotting of protein from SDS-PAGE

2.3.2.9.1 Preparation for Blotting

1) Prepare the transfer buffer (see appendix).

2) Following electrophoresis, equilibrate the gels in transfer buffer (Towbin buffer) about 15 minutes (for 0.75 mm thicken gel).

3) Cut the membrane to the dimensions of the gel. Wet the membrane by slowly sliding it at a 45° angle into transfer buffer and

allow it to soak for 15–30 minutes. Complete wetting of the membrane is important to insure proper binding.

4) Cut filter paper to the dimensions of the gel. Two pieces of extra thick filter paper per gel are needed for each gel/membrane sandwich. Completely saturate the filter paper by soaking in transfer buffer.

2.3.2.9.2 Assembly of the unit for standard transfers.

1) Remove the safety cover and the stainless steel cathode assembly.

2) Place a pre-soaked sheet of extra thick filter paper onto the platinum anode. Roll a pipette or test tube over the surface of the filter paper (like a rolling pin) to exclude all air bubbles. If thick or thin filter paper is used, repeat with one or two more sheets of buffer soaked filter paper.

3) Place the pre-wetted blotting media, polyvininylidene fluoride (PVDF), on top of the filter paper. Roll out all air bubbles.

4) Carefully place the equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane. Transfer would be incomplete if any portion of the gel was outside the blotting media. Roll out all air bubbles.

5) Place the other sheet of pre-soaked filter paper on top of the gel, carefully removing air bubbles from between the gel and filter paper. If thick filter paper is used, place two sheets on top of the gel, and remove bubbles from between each layer. If thin filter paper is used, place three sheets on top of the gel, and remove bubbles from between each layer.

6) Carefully place the cathode onto the stack. Presse to engage the latches with the guide posts without disturbing the filter paper stack.

7) Place the safety cover on the unit. Plug the unit into the power supply. Normal transfer polarity is cathode to anode, *i.e.*, red wire to red outlet and black wire to black outlet on the power supply.

8) Turn on the power supply. Transfer mini gels for 30 minutes at 15 V (5.5 mA/cm^2 for mini gels), it is recommended to prevent excessive heating during the run.

9) Following transfer, turn the power supply off, and disconnects the unit from the power supply. Remove the safety cover and the cathode assembly. Discard the filter paper. The transfer efficiency could be monitored by staining the PVDF with coomassie blue R-250 protein stain.

2.3.2.10 N-terminal peptide sequencing

1) Clean the working area with methanol and clean forceps and other sample handing devices with methanol. Did not wipe, but allow them to dry air.

2) Clean pipette tip with 50% methanol/0.1% trifluoroacetic acid

3) Wearing a non-powdered glove and cutting the PVDF membrane on interested lane of protein with new blade. The strip size about 2x2 mm.

4) Picked the strip of PVDF membrane into micro centrifuge tube and analyze the peptide sequencing by Procise® CLC protein sequencer (PE Applied Biosystems).