Chapter 3

Material and Methods

1. Materials

1.1 Experimental plants

The experiments were conducted by using one year layering propagated longans cv.Dor grown with fine sand in 12 inches diameter pots. The major elements for these trees provided in form of a concentrated solution, which composed of Mg^{2+}, K^{+}, Ca^{2+}, SO_4^{2-}, $H_2PO_4^{-}$ and NO_3^{-} at 5 meq/l for each element, pH = 6.5. The minor elements used followed that advice of Hoagland and Arnon (1952). The mixture of major and minor elements nutrient solution was given almost everyday but stop applied it just before started treating with potassium chlorate. The trees were grown in the experimental plot at the Department of Horticulture, Chiang Mai University (latitude 18° 47 / N, 312 m asl), Thailand during November 2000 to April 2002.

1.2 Other apparatus

1.) Electronic balance

2.) Oven and desiccator

3.) Electronic blender

4.) Spectrophotometer HITACHI model U-2001

5.) Atomic absorption spectrophotometer PERKIN ELMER model 3100

6.) Photosynthetic measurement apparatus LICOR model Li-6200

7.) Vacuum rotary evaporator

8.) Gas chromatography SHIMADZU model GC-148

2. Experimental design

There were four experiments in this study.

2.1 **Experiment 1** Study on the appropriate concentration of $KClO_3$ for induces flowering in one year- old longans, four concentrations of $KClO_3$, 0, 0.05, 0.10 and 0.15 g/ l/ pot in

randomized complete block design (RCB). There were 3 replications in each treatment. The best treatment was being used in the experiment 3 and 4.

2.2 **Experiment 2** Study on root growth, two factors in RCB were employed. Trees were grown in hydroponics with 4 replications. Four liters of 250 ppm potassium chlorate solution were applied, compared with control. One plant was used for an experimental unit.

2.3 **Experiment 3** Study on root respiration, potassium chlorate 0.05 g/l was applied to each plant in RCB, 3 replications compared with control. Three plants were used for an experimental unit.

2.4 **Experiment 4** Study on some physiological and chemical changes in roots, leaves and shoots after applied with 0.05 g/l $KClO_3$ in RCB, 3 replications compared with control. Three plants were used for an experimental unit.

3. Methods

3.1 Experiment 1

Trees were grown in pots filled with fine sand. Treated with various concentrations of $KClO_3$ and recorded the following data,

a. The date of visible flower bud.

b. The percentage of flowering.

c. The numbers of staminate and pistillate flowers.

3.2 Experiment 2

Trees were grown in hydroponics, new roots lengths were collected every week until it turn brown.

3.3 Experiment 3

Roots about 20 g were washed then placed in to a closed plastic box with known volume, left for 1 hour. The carbon dioxide was measured once a week for four weeks.

3.4 Experiment 4

The physiological aspects

a. The photosynthetic rate and stomatal conductance; the measurement was using Li-6200 Portable Photosynthesis system (Li-COR. Inc.). The young fully expanded

leaves were measured once a week for four weeks at 08.00, 10.00, 12.00, 14.00 and 16.00 o'clock.

b. Stomatal behaviors during the day observed by silicone rubber impression method (Tunsuwan and Büneman, 1973) using the center of the lower surface of the young fully expanded leaves weekly, every two hours from 08.00 a.m. to 04.00 p.m. Printed the surface of silicone rubber replicas on microscope slides by flooding the slide surface with the mixture of acetate paper and acetone solution, then pressed the silicone rubber replicas on the slide.

c. Electrolyte leakage (EL) of leaves and roots were modified from McKersie *et al.* (1996), Maier and Lang (1994) and Ruter (1996). Ten leaves discs (0.8 cm in diameter) from the 3rd to 6th sampling leaves and 0.5 g of 1 cm excised young root were placed separately in the test tubes, each containing 10 ml deionized water. Induced electrical conductivity (EC) readings of leaves and roots were measured after 1 hr of incubation at room temperature. To determine potential electrical conductivity, tubes were held at 80°C in a water bath for 1 hr. After samples were placed at 27°C overnight, potential EC was measured. Percentage of EL was calculated, induced EC x 100 / potential EC.

The chemical aspects

a. Chlorophyll a and b content of the leaves were analyzed followed by Whitham *et al.* (1971). The optical density of the samples were measured by spectrophotometer at wave-length 645 and 663 manometers, calculated and reported in milligrams of chlorophyll per gram fresh weight of leaves. Some leaves were left at the room temperature to measure chlorophyll degradation for another two consecutive days.

b. Peroxidase activity was determined by using three grams of root and shoot samples, grind in a cold motor with 2 ml of pH 4.5 citrate phosphate buffer and liquid nitrogen. Placed the material in a centrifuge tube and washed the residue out of the motor with an additional 1 ml of buffer into the centrifuge tube. Centrifuge at 6000 x g for 10 min, poured the supernatant in another centrifuge tube. Resuspend the pellet in 3 ml of buffer and centrifuge at 6000 x g for 10 min, discarded supernatant. Resuspend the pellet in 3 ml of 0.2 M $CaCl_2$ and centrifuge at 10,000 x g for 10 min.

The supernatant was poured together with the first one. The enzyme was kept cold at all times during the separation. Peroxidase was assayed by placing 0.5 ml of citrate phosphate buffer pH 4.5 in a test tube, added with 0.5 ml of 1500 ppm of o- phenylenediamine and 1 ml of a 3% solution of hydrogen peroxide. Placed 0.5 ml of sample extracted enzyme in a cuvette prior to reading the absorbance at 492 nm on the spectrophotometer. Heat denatured enzyme (boiling water 5 min) was used as a blank. Calculated the activity of peroxidase by the following formula.

$$C = (A_1 - A_2)/ d*\varepsilon*b*t$$

C = the activity of enzyme (μmole/ ml/ min or unit/ ml)

A_1 = absorbance at the time t_1

A_0 = initial absorbace at the time t_0

d = dilution value

ε = molar extinction coefficient ($mM^{-1}cm^{-1}$)

b = cuvette width (cm)

c. Analysis of carbohydrate, five cm long of terminal shoot samples, mature leaves at 3rd to 6th leaf position from the apex and root were sampling, oven dry at 70 ^{0}C for 72 hours, grind to powder to determine for the content of carbohydrate in shoot, leaf and root. Extraction of total non-structural carbohydrates (TNC) was using the acid extraction method described by Smith *et al.* (1964) and modified by Chaitrakoolsup and Subhadrabandhu (1983). Extraction of reducing sugars (RS) were using the ethanolic extraction method described by Yemm (1935). Using Nelson's reducing sugar procedures expressing the amount of carbohydrates as mg D-glucose equivalent.

d. Nitrogen content was determined in horticultural laboratory. Roots (less than 0.5 mm in diameter), leaves (3th to 5th actively leaf) and shoots (5 cm from shoot tip excluded leaf petiole) were sampling, washed with distilled water and oven dry at 70 ^{o}C for 72 hours and grind to powder. Wet Acid Digestion, modified by Ohyama

et al. (1985, 1986) was employed. Each 0.05 g of sample was added with conc. H_2SO_4 in test tube, covered with parafilm, overnight. Digested at 180 ^{0}C for 10 min, let it cool down. Added 0.3 ml of H_2O_2, vortex and digested at 230 ^{0}C for 30 min. If the solution could not be seen transparency, 0.2 ml of H_2O_2 was added and digested again. After the tubes were cool down, 5 ml of distilled water was added, left overnight. The digested solution was adjusted to 50 ml with distilled water, kept in plastic bottle at room temperature. These sample solutions would be used to determine for nitrogen and phosphorus. Four reagents were used for nitrogen determination. Reagent A, 25 g of EDTA.2 Na was dissolved in 500 ml of distilled water, adjust pH to 10. Added 20 ml of methyl red, stirred, filled up with distilled water to 1,000 ml. Reagent B, 136.09 g of KH_2PO_4 and 2.75 g of benzoic acid were dissolved in distilled water and made up to 1,000 ml. Reagent C, 100 mg of sodium nitroprusside was dissolved in distilled water, phenol 10.25 ml was added then filled up to 1,000 ml with distilled water, kept in the fridge. The solution should be used within two weeks. Reagent D, 10 g of NaOH, 7.06 g of $Na_2HPO_4 .7H_2O$ and 31.8 g of $Na_3PO_4 .12H_2O$ were dissolved in distilled water, 10 ml sodium hypochlorite was added, filled up with distilled water to 1,000 ml. Standard solutions were prepared from $(NH_4)_2SO_4$, 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l. Standard solution or sample solution 0.5 ml was added with 0.5 ml of reagent A and B, a drop of 1 N NaOH and 2.5 ml of reagent C and D were added respectively. Adjust the solution to 25 ml, left at 30 ^{0}C for 3 hours, the absorbance was record by spectrophotometer at 625 nm compared with standard curve.

e. Nitrate content was determined by Colorimetric method. Oven-dried samples were slowly extracted with 80% ethanol, adjusted the volume to 25 ml. $NaNO_3$ 5 mM was pipette 0, 10, 20, 30, 40 and 50 µl to each test tube for standard, 20 µl of sample solution for samples and 20 µl of 80% ethanol for a blank. All of them were diluted with distilled water up to 50 µl. Two hundred µl of 5% salicylic acid was added to standard and sample, but 200 µl of H_2SO_4 was added to a blank, vortex and left for 20 min at room temperature. Five ml of 2N NaOH was added, vortex, wait for 20 min then the absorbance was measured by spectrophotometer at 410 nm.

f. Phosphorus was determined by Colorimetry method. Three reagents were prepared. Reagent A, 25 g of $(NH_4)_6Mo_7O_{24}$ was dissolved in distilled water and filled up to 200 ml. Reagent B, 200 ml of sulfuric acid was diluted with 200 ml of distilled water, overnight then made it up to 500 ml. Reagent C, slowly poured reagent A into reagent B, over night, adjusted to 1,000 ml with distilled water then kept in the dark. Stanous chloride solution was prepared by dissolved 0.25 g of $SnCl_2 .2H_2O$ in 5 ml of HCl, 20 ml of distilled water was added. KH_2PO_4 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l was used as standard. Sample solutions (the same digested solutions for determining nitrogen content) 0.5 ml was added with a little of distilled water. Reagent C, 1 ml and 0.2 ml of stannous chloride were added respectively, adjusted the volume to 25 ml with distilled water and left for 15 min. Absorbance was recorded with spectrophotometer at 660 nm, compared with standard.

g. Potassium determination was digested followed by Mizukoshi *et al.* (1994). Samples were dried, grind to power and weighed 0.5 g each. $HClO_4$ 0.5 ml and HNO_3 0.5 ml were added respectively, vortex then the tubes were covered with parafilms overnight. Digested at 100 °C to let out a yellow smoke of NO_2^-. The temperature was increased to 210 °C until the solution was dried, let it cool down. Diluted HCl (1 HCl : 4 H_2O_2), 1 ml was added, vortex then placed back to the digestion box at 100 °C, 5 min and let cool. Adjust the volume to 50 ml with distilled water, kept in plastic bottle at room temperature. Each sample solution 0.5 ml was diluted to 25 ml with distilled water. Concentration of potassium was measured by atomic absorption spectrophotometer at 766.5 nm compared with potassium standard 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l.

h. Auxin and gibberellin (GA) like-substances extraction and purification were modified from Scheider *et al.* (1972). Twenty gram fresh weights of roots were used for each analysis. Roots were homogenized in a blender in 40 ml of 95% (v/v) methanol. The mixture was kept at 4 °C for 18 hours, filtered, the procedure was repeated twice. The filtrate was evaporated in vacuum rotary evaporator at 35°C until dry. Dissolved with 15 ml 0.5 M, pH 8.0 sodium phosphate buffer. Fractionated by extraction into 20 ml, 100% ethyl acetate in separatory funnel. Water phase was

acidified by 6N HCl to pH 2.5, extracted with 20 ml, 100% ethyl acetate for five times. Discarded the water phase, ethyl acetate phase was collected together, evaporated until dry. Dissolved with 1 ml, 95% methanol then purified by paper chromatography. Filtered paper, Whatman no. 1, 9 x 28 cm was stripped with 200 µl of extracted solution containing GA or IAA-like substances at 2 cm above the lower end, let dried. Dipped the lower end of paper just below the stripped line into the eluent containing 99.7% isopropanol: 25% NH_4OH: distilled water, 10: 1: 1 (v/v) in developing chamber. After the front solvent reached to18 cm above the stripped line, took off and dried at the room temperature. These paper chromatograms (PC) were divided in to ten portions, R_F value 0.1 to 1.0. Under stripped line was R_F 0.0 (control). Each R_F PC was added with 3 ml, 50% acetone (v/v) to dissolve GA and IAA like-substances from PC.

Rice Micro-drop Bioassay (RMB), modified from Nishijima *et al.* (1993) was employed for GA bioassay. Taichung Native 1, dwarf type rice was washed in 10% sodium hypochlorite for 10 minutes, rinsed well with distilled water. The seeds were soaked in 20% uniconazole overnight, washed five times with distilled water then sowed in plastic box for 2 days at 28± 2° C (about 2 mm coleoptiles were observed). Eight small seedlings transferred to a 4-oz. bottle containing 20 ml, 8% agar, placed under 2,000 lux, 28± 2° C for 72 hours. Applied 2 µl of each R_F solution between coleoptile and the second leaf, placed them back to the same condition for three days. The secondary leaf sheaths were measured in cm. Good results of R_F values were selected to quantify for GA from each sample by repeated the procedure again then compared with standard curve.

Indoleacetic acid and adventitious root was employed as auxin bioassay modified from Witham *et al.* (1986). Uniform cuttings were made from eight-day-old mung bean seedings. Cuttings were ready to assay when the primary leaves were fully expanded and the trifoliate bud had not expanded. Each cutting consisted of a 3 cm hypocotyl, epicotyl, primary leaves and apical meristem. Five cuttings were placed into 0.5 ml of standard IAA (0, 5, 10 µg) or the solution of 497 µl of distilled water and 3 µl of each sample in a 15 x 100 mm test tube. After the initial

solution was absorbed, about three hours added distilled water and maintain the water level at the cotyledonary node during the five-day rooting period at 28±2° C, and 16-hour photoperiod with about 2,000 lux. After fives days, count the roots and record the average number of roots per cutting. IAA content of samples was calculated from IAA standard curve.

To reduce the variation from bioassay, determination of auxin and auxin-like substances by spectrophotometry was employed. IAA (MW= 175.19) was used as IAA standard, the concentration were 0, 0.1, 1.0, 10, 20 and 30 ppm. One ml of each standard concentration and sample was pipette into test tube, the sample solution contained 10 µl of extracted sample and 990 µl of 50% ethanol. Two ml of Salkovski reagent (0.5 M $FeCl_3$ 1 ml + 35% $HClO_4$ 50 ml) was added into the tube, mixed well, incubated at 30° C, dark for 30 min. Standards and samples were measured by spectrophotometer at 530 nm. The auxin and auxin-like substances then calculated from IAA standard curve.

h. Ethylene was determined by Submerged vacuum extraction (Saltveit, 1982). The method of extracting internal gas under the surface of an aqueous, saturated ammonium sulfate was used to minimize the solubility of gas into water. Twenty grams of root was cut about 1 cm long, rapidly placed under the container filled with saturated ammonium sulfate. When vacuum was applied and release, there was a mass flow of gas out of the sample through the lenticels, pores and other regions of low resistance. Gases dissolved within the tissue will come out of solution as the vacuum was applied. The gas was collected by syringe and quantifies by gas chromatography at 150 °C injector, 120 °C of oven, column and detector. Flow rate of nitrogen was 60 ml/min and 50 k Pas for hydrogen and air. The concentration of ethylene was calculated compared with standard ethylene.

i. Cytokinin-like substances extraction and purification were modified from Chen *et al.*, 1997. Twenty-five gram fresh weights of roots were used for each analysis. Roots were homogenized in a blender in 250 ml of 80% (v/v) ethanol. The mixture was kept at 4 °C for 17 hours, filtrated and evaporated in vacuum rotary evaporator at 45°C until the volume decreased to 50 ml. The solution was adjusted to pH 2.5

and fractionates by extracted in 30 ml of 99.8% ethyl acetate. The water phase was passed through a column (1 x 25 cm) of Dowex resin 50W x 8-100 (50-100 mesh), a strong acidic cation exchange ion form with the flow rate 2 ml per minute. The column was washed well with 20 ml of distilled water with the same flow rate followed by 20 ml of 70% ethanol and another 10 ml of distilled water. Continuous flowing was used to prevent air bubble happened in the column. Discarded the solution from the column and then eluted with 20 ml of 5 N NH_4OH with 0.5 ml per minute flow rate followed by 10 ml of distilled water. The solution was collected and evaporated at 45 ^{0}C until it almost dry. Washed the evaporatory flask with 80 % ethanol. About 1 ml of solution was collected for paper chromatography. The column was washed well with 2 N HCl and 100 ml of distilled water with the flow rate 2 ml per minute then the column was ready for the next sample. Three hundred micro liter of the solution was applied as a 9-cm strip at 2 cm above the lower end of 9 x 28 cm Whatman paper no. 1. The eluent in a developing chamber was 95% of isopropanol : 25% NH_4OH : H_2O (10: 1: 1, v/v). Let the chromatogram dry after the elution reached the solvent front (18 cm) then divided the chromatogram from the strip line to the solvent front into ten. R_F 0 was the portion under the strip line, R_F 0.1 was the portion next to R_F 0 and R_F 1.0 was the top portion. Each portion was test for cytokinin like substances by bean hypocotyl bioassay, modified from Manos and Goldthwaite (1976). Media from 30% w/v of sugar (sucrose) and 10% w/v of agar was added to test tube (25 x 150 mm) to make a height of 40 mm. Tubes were covered with plastic caps, autoclaved at 121 $^{\circ}$C for 15 min, and allowed to cool. Soybeans (*Glycine max*) were surface sterilized in a 10% sodium hypochlorite solution for 15 min with occasional stirring, then rinsed 5 times with sterile distilled water in transfer cabinet. One soybean was embedded in each tube, and the tubes were placed in the dark for about 7 days, when most hypocotyls had elongated to about 100 mm (giving about 50 tissue sections per seedling). A plastic mm ruler sterilized with 80% ethanol was placed under a sterile glass Petri dish (150 x 20 mm). A hypocotyl from one seedling was excised with a sterile razor blade above the primary root and just below the cotyledons. It was placed in the Petri dish and

2-mm sections were cut from the entire hypocotyl. Six sections were randomly placed on a glass bottle of sterile media, 10 ml of soybean callus Miller (1963) and small pieces of R_F paper chromatograph contained cytokinins to be tested. The glass bottles containing hypocotyl sections were incubated at 25-28° C, 2,000 lux of light intensity for 13 days. The hypocotyl sections in each bottle were weighed on an analytical balance sensitive to 0.1 mg. The R_F which gave the best result were repeated the procedure again and cytokinin content was calculated compared with the standard zeatin.