Chapter 3

Materials and Methods

1. Materials

1.1 Experimental plants

One year old air-layered longans cv. Daw, were grown with fine sand and rice husk ash (1:1) in 5×12 inches diameter plastic bags. These trees were applied with Doi Khum 1 and 2 nutrient solutions, pH 5.5-6.0 every three days until started treating with potassium chlorate (Figure 1). The experiments were conducted during January 2005 to December 2007 at the greenhouse of Department of Horticulture, Faculty of Agriculture, Chiang Mai University, Thailand.

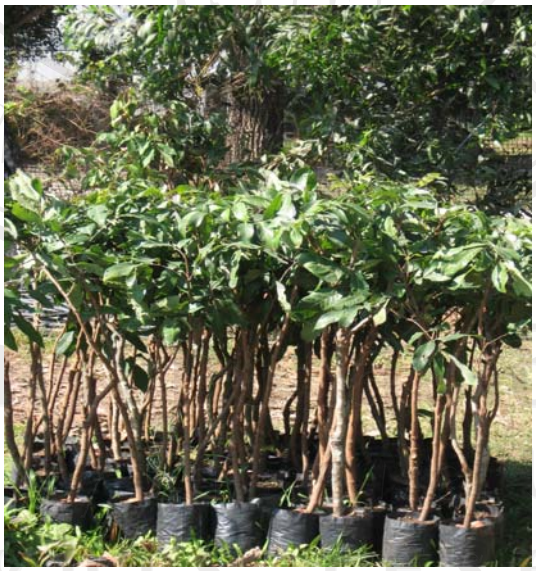

Figure 1 One year-old air-layered longans cv. Daw which use as experimental plants

1.2 Other apparatus

1.) Electronic analytical balance, Model 211S, Sartorious, Germany

2.) Oven and desiccator

3.) Electronic blender

4.) Spectrophotometer, Jenway 6300, Jenway, England

5.) Atomic absorption spectrophotometer, Model 3100, PERKIN ELMER, USA

6.) Vacuum rotary evaporator

8.) Freezer, refrigerator. Model SF-C992, Sanyo, Japan

9.) Gas chromatography, model GC-14B, SHIMADZU, Japan

2. Methods

2.1 Experiment 1 The concentration effect of potassium chlorate on flower induction of derooted air-layered longan cv. Daw

The derooted air-layered longans were grown in a non-shading greenhouse which the relative humidity was controlled by 15 min. interval automatic water sprayer during 9.00 am - 5.00 pm (Figure 2). The experimental design was completely randomized design (CRD). There were four replications in each treatment and ten plants were used for an experimental unit.

2.1.1 Cultured in water

2.1.1.1 Derooted air-layered longans were dipped at high concentrations of $KClO_3$ i.e. 0, 1,000, 2,500, and 5,000 ppm for 24 h followed by culturing in water

2.1.1.2 Derooted air-layered longans were dipped at low concentrations of $KClO_3$ i.e. 0, 100, 200, 300, 400 and 500 ppm for 24 h followed by culturing in water

2.1.2 Cultured in nutrient solution Derooted air-layered longans were dipped in nutrient solution Doi Khum 1 and 2 in ratio 1:1 treated with low concentrations of $KClO_3$ i.e. 0, 100, 200, 300, 400 and 500 ppm for 24 h followed by culturing in the same nutrient solution.

The date of visible flower bud and the percentage of flowering were recorded. The best treatment was used in the experiment 2 and 3.

Figure 2 Non-shading greenhouse with automatic water sprayer

2.2 Experiment 2 Effect of potassium chlorate ($KClO_3$) on flowering and morphological change of the terminal buds in air-layered and derooted air-layered longans cv. Daw

The root of air-layered longans were washed with water. After that the rooted and derooted air-layered longans were dipped in 500 ppm $KClO_3$ solution (pH 5.5-6.0) for 24 h, and then the air-layering were grown with fine sand and rice husk ash (1:1) in 12.7x30.48 cm plastic bags, but the derooted were cultured in 1.25 liter plastic bottle containing water. Both of them were kept in the greenhouse with the same condition as experiment 1. There were four treatments with four replications. Five plants were used for an experimental unit.

Treatment 1 air-layered longans without $KClO_3$ treatment (R)

Treatment 2 air-layered longans with $KClO_3$ treatment (R+ $KClO_3$)

Treatment 3 derooted air-layered longans without $KClO_3$ treatment (DR)

Treatment 4 derooted air-layered longans with $KClO_3$ treatment (DR+$KClO_3$)

The shoots tips, 0.2-0.3 cm in length, were sampling at 10, 15, 20 and 25 days after treatment. They were stopped activity by FAA fixation. Then the shoot tips samples were dehydrated in graded alcohol series. After that, temporarily stained tissues with TBA plus absolute alcohol and erythrosine to made them visible in the paraffin block, and then transferred to pure TBA infiltration 3 times in 12 h interval. They were transferred into solution of TBA mixed with paraffin oil (1:1) and pure paraffin oil for each 12 h, respectively. Finally, the tissue samples were kept in paraplast solution at 55-60 $^{\circ}$C for 1-2 months and embedded in paraffin blocks. The sample blocks were sectioned by rotary microtome, and observed the morphological change of the terminal buds using microscopy. For frozen section, the shoot tips samples were fixed by FAA and then embedded in frozen medium at -20 $^{\circ}$C. After that the frozen samples were sectioned by freezing microtome.

2.3 Experiment 3 Effect of potassium chlorate on physiological and biochemical changes in leaves and shoots of air-layered and derooted air-layered longan cv. Daw.

The experimental plants were grown in the same condition as experiment 2. The experimental design was CRD, four treatments with four replications. But 10-20 plants were used for an experimental unit. The leaves and shoots were sampling at 0, 5, 10, 15, 20 and 25 days after treatment for physiological and biochemical analysis.

2.3.1 The chlorophyll a, b and total chlorophyll content

The mature fully expanded leaves at 2nd compound leaves position were analyzed by chlorophyll extraction method modified from (Witham *et al.*, 1971). Ten leaf disks (3 mm in diam.) were punched out from the leaf blade and weighed, the

chlorophyll was extracted by 10 ml 80% acetone. The disk samples were incubated in the dark at room temperature for 48 h. Then the optical density of extracted solution was obtained by spectrophotometer at 645 and 663 nm and chlorophyll contents were calculated as $mg.gFW^{-1}$ by the following equations:

$$\text{Chlorophyll a} = \frac{(12.7 \times D_{663} - 2.69 \times D_{645}) \times V\ (ml)}{(1{,}000 \times gfw)}$$

$$\text{Chlorophyll b} = \frac{(22.9 \times D_{645} - 4.68 \times D_{663}) \times V\ (ml)}{(1{,}000 \times gfw)}$$

$$\text{Total Chlorophyll} = \frac{(20.2 \times D_{645} + 8.02 \times D_{663}) \times V\ (ml)}{(1{,}000 \times gfw)}$$

D_{663} = Absorbance at 663 nm

D_{645} = Absorbance at 645 nm

gfw = gram fresh weight (g)

V = Total extract solvent (ml)

2.3.2 Carbohydrate analysis

The terminal shoot with five cm in length and mature leaves at 2nd to 4th compound leaves position from the apex were sampling, cleaned with deionized water and dried at 70 °C about 72 h until constant dry weight. After drying, the samples were ground and pass through 40-20 mesh screen, thoroughly mixed and stored in conditions that maintain sample dryness. If samples absorbed moisture during grinding, additional drying was applied prior to weighing for analysis of carbohydrates and nutrient. Extraction of total nonstructural carbohydrates (TNC) used modified acid extraction method (Smith *et al*., 1964; Chaitrakulsup and Subhadrabandhu, 1983). Whereas total sugar (TS) was extracted by the method described by Khalafalla and Palzkill (1990), and the extraction method described by Dubois *et al*. (1956) was used for extraction of reducing sugars (RS). The amount of carbohydrates (TNC, TS and RS) were determined by Nelson's reducing sugar procedures (Hodge and Hofreiter, 1962).

2.3.3 Nutrient analysis

2.3.3.1 Nitrogen content

Shoots (5 cm long excluded leaf petiole) and leaves (2^{nd} to 4^{th} compound leaves position) were collected. Sample preparation was the same procedure as carbohydrate analysis. Acid digestion, modified by Ohyama *et al.* (1985, 1986) was employed. Each 0.05 g of dried sample was added with conc. H_2SO_4 in test tube and covered with parafilms before lefting overnight. They was digested at 180 $^{\circ}$C for 10 min and then let it cool down. After that they was added with 0.3 ml of H_2O_2, and digested at 230 $^{\circ}$C for 30 min. If the solution could not be clear, 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, and 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 nitrogen and phosphorus. Four reagents were used for nitrogen determination. Reagent A, 25 g of EDTA·2Na was dissolved in 500 ml of distilled water and adjusted pH to 10 with 10 N NaOH. After that it was added with 20 ml 0.1% methyl red and adjusted the volume to 1000 ml with distilled water and then kept in the dark. Reagent B, 136.09 g of KH_2PO_4 and 2.75 g of benzoic acid were dissolved in distilled water and filled up to 1000 ml. Reagent C, 0.1 g of sodium nitroprusside was dissolved in distilled water, added with 10.25 ml phenol and then filled up to 1000 ml with distilled water, kept in 4 $^{\circ}$C. It should be used within two weeks. Reagent D, 10 g of NaOH, 7.06 g of $Na_2HPO_4 \cdot 7H_2O$ and 31.8 g of $Na_3PO_4 \cdot 12H_2O$ 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 $mg \cdot L^{-1}$. 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. The solutions was adjusted the volume to 25 ml with distilled water and kept at 30 $^{\circ}$C for 3 h. The absorbance was recorded by spectrophotometer at 625 nm compared with the standard curve.

2.3.3.2 Nitrate content

Nitrate content was determined by colorimetric method (Cataldo *et al*., 1975) The leaf samples at 1st compound leaves position were ground in liquid nitrogen and slowly extracted with 80% ethanol 3 times by centrifugation at 10000 rpm at 4 °C for 15 min. The supernatant was collected and adjusted the volume to 25 ml then kept in plastic bottle at 4 °C. KNO_3 (25 µmole·ml^{-1}) standard solution was prepared and diluted to 2.5 µmole·ml^{-1}, and then pipetted 1, 2, 3, 4 ml to each test tube, adjusted with distilled water up to 10 ml for standard which the concentration of standard was 0.25, 0.5, 0.75 and 1.0 µmole·ml^{-1}respectively .After that 0.05 ml of sample solution for samples and 0.05 ml of 80% ethanol for a blank was used, 0.2 ml of 5% salicylic acid (in conc.H_2SO_4) was added into standard and samples, but only 0.2 ml of conc. H_2SO_4 into a blank.The solutions were mixed together and left to cool down for 20 min at room temperature. Finally, 2N NaOH 4.75 ml was added and mixed well, wait for 20 min then the absorbance was measured by spectrophotometer at 410 nm.

2.3.3.3 Phosphorus content

Phosphorus content was determined by colorimetric method (Mizukoshi *et al*., 1994) 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, 250 ml of sulfuric acid was diluted with 200 ml of distilled water, left overnight then filled it up to 500 ml. Reagent C, slowly poured reagent A into reagent B, left over night, adjusted to 1,000 ml with distilled water then kept in the dark. Stannous chloride solution was prepared by dissolved 0.25 g of $SnCl_2 \cdot 2H_2O$ in 5 ml of HCl, 20 ml of distilled water was added. KH_2PO_4 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 1.5 and 2.0 mg·L^{-1} was used as standard. Sample solutions 0.5 ml, extracted with acid digestion together with nitrogen content, 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. The absorbance was recorded with spectrophotometer at 660 nm and quantified by comparing with the standard.

2.3.3.4 Potassium content

Potassium content was determined by colorimetric method (Mizukoshi *et al*., 1994). Samples were dried, ground to powder 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 $^{\circ}$C to let out a yellow smoke of NO_2^-. The temperature was increased to 210 $^{\circ}$C until the solution was dried, let it cool down. Diluted 1 ml HCl with 4 ml distilled water, after that it was added into the test tube, vortex and then placed back to the digestion box at 100 $^{\circ}$C, 5 min and cool down. Adjusted the volume to 50 ml with distilled water, kept in plastic bottle at room temperature for potassium and calcium analysis. For potassium analysis, 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 $mg \cdot L^{-1}$.

2.3.3.5 Calcium content

Calcium content was also determined by colorimetric method (Mizukoshi *et al*., 1994). Lanthanum solution was prepared by dissolving in 500 ml of distilled water added 10 ml of 37% HCl and then adjusted volume to 1,000 ml. Each extracted solution 1 ml from 2.3.3.4 was diluted to 25 ml with lanthanum solution. After that the absorbance was measured by spectrophotometer at 442.7 and 285.2 nm, respectively and compared with calcium standard (0, 1, 2, 3, 4 and 5 $mg \cdot L^{-1}$) which prepared by using $CaCO_3$, added with 10 ml of lanthanum solution and then adjusted to 100 ml with 0.5 NH_4Cl.

2.3.4 Nitrate reductase activity (NRA)

Nitrate reductase activity was determined by colorimetric method modified from Jaworski (1971). A 0.5 g of leaf samples were taken from the 1st fully expanded compound leaves and then chopped into small pieces, It was filled in a test tube containing 5 ml incubating solution (25 mM phosphate buffer[pH 7.3], 10 mM KNO_3 and 0.05 mM EDTA) and then sealed and kept in the dark for 2 h at room temperature. A 0.4 ml aliquot was taken from the tube with a pipette, mixed with 0.3

ml 0.02% NEDA (n-1-napthal-ethylene-diamine dihydrochloride) and 0.3 ml 1% sulfanilicacid (sulfanilamide), added 4 ml distilled water.While the blank was contained with 0.4 ml phosphate buffer, 0.3 ml 0.02% NEDA, 0.3 ml 1% sulfanilamide and 4 ml distilled water. The absorbance at 540 nm was read on a Jenway 6300 spectrophotometer compared with $NaNO_2$ standard curve

2.3.5 Hormonal analysis

2.3.5.1 Indole acetic acid (IAA)

After the samples were collected the leaf and shoot samples were immediately frozen by liquid nitrogen and stored in a refrigerator at -80 °C until analysis. For extraction, each 5 g samples were seperately ground in liquid nitrogen by using motar and pestle and extracted with 95% (v/v) cold methanol at 4 °C for 18 h and then filtered. The procedure was repeated 2 times. After that the filtrate was evaporated to dryness at 35 °C and dissolved with 15 ml of 0.5 Msodium phosphate buffer (pH 8.0). Fractionated by extraction into 20 ml, 100% ethyl acetate in separatory funnel. Water phase was acidified by 6 N HCl to pH 2.5 and repeatedly extracted for four times. The ethyl acetate phase was collected together and evaporated at 35 °C until dry. It was dissolved with 1 ml 95% methanol then purified by paper chromatography. Whatman No. 1, 9 x 28 cm was stripped with 200 µl of extracted solution containing IAA-like substances at 2 cm above the lower end, let dried. The lower end of paper was dipped just below the stripped line into the mobile phase containing 99.7% isopropanol, 25% NH_4OH and distilled water in ratio 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 chromatography were divided into ten portions, R_f value 0.1 to 1.0. Under stripped line was R_f 0.0 (control). Each Rf paper chromatography was added with 3 ml of 50% acetone (v/v) to dissolve IAA like-substances.

The extracted samples were used to determine IAA contents by spectrophotometry (Minamisawa *et al*., 1992). 10 mM IAA (MW= 175.19) in 50% methanol was used as IAA standard. It was diluted to 1 mM for preparing standard series at the concentration of 0, 10, 20, 50, 100 and 150 µM. One ml of each

standard concentration and sample was pipetted into test tube, the sample solution contained 10 µl of extracted sample and 990 µl of 50% methanol. Two ml of Salkovskii reagent (0.5 M $FeCl_3$ 1 ml + 35% $HClO_4$ 50 ml) was added into the tube, mixed well, incubated at 30 °C in darkness for 30-60 min. Standards and samples were measured by spectrophotometer at 530 nm. The IAA contents was calculated from IAA standard curve as $\mu M \cdot gFW^{-1}$.

2.3.5.2 Gibberellin like-substances (GAs)

The same extraction method as IAA was used for GAs analysis. R_f 0.3-0.8 of paper chromatography which gave good results for GAs activity was cut into small pieces and put in 6x4x3.5 cm plastic box containing 5 ml of 0.01 potassium phosphate buffer, pH 5.0. Rice Secondary Leaf Sheath Bioassay (RSLSB), modified from Wangsin (2002) was employed for GAs bioassay. Seeds of Phrae 1 rice were sterilized with 5% sodium hypochlorite for 15 minutes, rinsed well with sterilized distilled water for three times. After that they were germinated in plastic box for 3 days at 28± 2 °C in darkness. When the coleoptiles were about 4-5 mm in length, ten seedlings were planted in the plastic box containing extracted GAs that mentioned above and incubated for 7 days, at 28± 2 °C under continuous irradiation (115.38 $watt \cdot m^{-2}$). Secondary leaf sheath of rice was measured for quantifying gibberellin-like substances by comparing with GA_3 standard curve.

2.3.5.3 Cytokinin-like substances

The extraction and purification methods were modified from Chen *et al*. (1997) and Wangsin (2002). Five grams fresh weight of shoots or leaves were homogenized with motor in 200 ml of 80% (v/v) ethanol. The mixture was shake well and kept at 4 °C for 17 h. After that It was 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 with 6 N HCl and partitioned by extracting in 30 ml of 99.8% ethyl acetate, the water phase was collected. The column of (1 x 25 cm) Dowex resin 50W x 8-100 (400 mesh), a strong acidic cation exchange ion, was washed well with 100 ml of distilled water with the flow rate 2 ml per minute two times. The water

phase 10 ml was passed through a column with the same flow rate, 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 20 ml of distilled water. Continuous flowing was used to prevent air bubble happened in the column. The elution was collected and evaporated at 45 °C until almost dry. After that the extracted solution was lenched out with 80% ethanol, and collected about 1 ml of solution for paper chromatography. The column was washed well with 2 N HCl 20 ml, followed by 100 ml of distilled water 2 times then the discarded solution was repeatedly passed through the column two times. The extracted solution was stripped at 2 cm above the lower end of 9 x 28 cm Whatman paper no. 1, and developed in 95% of isopropanol : 25% NH_4OH : H_2O (10:1:1, v/v) about 9 h. After the elution reached the solvent front (18 cm), the chromatogram was left to dryness and then divided it from the strip line to the solvent front into ten portion (R_f 0 to R_f 1.0). Each portion was tested for cytokinin-like substances.

The soybean hypocotyl bioassay, modified from Manos and Goldthwaite (1976) was used to test for biological activity of each portion. Soybeans (*Glycine max*)(SJ 5) were surface sterilized in 70% ethanol for 5 min followed by 10% sodium hypochlorite solution for 15 min, then rinsed with sterile distilled water in aseptic condition. One soybean was embeded in each test tube (25×150 mm) that filled with 30% w/v of sugar (sucrose) and 10% w/v of agar, and the tubes were incubated for about 7 days in darkness until most hypocotyls had elongated to about 10 cm. A hypocotyl from one seedling was excised above the primary root and just below the cotyledons and then 1 mm sections were cut from the entire hypocotyl. Six sections were randomly placed in the bottle containing soybean callus media (Miller, 1963) and small pieces of each R_f paper chromatograph portion contained cytokinins to be tested. They were incubated at 28± 2 °C, for 13 days in darkness. At the end of the incubation period, each section was weighed on an analytical balance sensitive to 0.1 mg. The R_f 0.4-0.9 which gave the best result were repeated the procedure again and cytokinin-like substance contents was calculated compared with the standard kinetin.

2.3.5.4 Ethylene

Ten shoots (5 cm in length) were cut, and then rapidly put into 50 ml centrifuge tube which were punched hole on lid and closed with silicone and sealed with parafilm. The centrifuge tubes were then incubated at 20 ± 2 ^{o}C for 3 h. For leaves, ten leaves cut from 2nd compound leaves position were sampling and put into plastic box and sealed with parafilm. Ethylene was quantified by using a gas chromatography at 150 $^{\circ}$C injector, 55 $^{\circ}$C column oven and 150 $^{\circ}$C FID detector. Flow rate of nitrogen carrier gas was 60 ml·min^{-1} and 50 k Pas for hydrogen and air. The concentration of ethylene was calculated compared with standard ethylene.