Chapter 3

Materials and Methods

This study was aimed to study the possibility to use potassium chlorate with paclobutrazol for inducing flowering, some influences factors and some biochemical changes during a flower induction period in lychee cv. Kom, Chakapad and Hong Huay. This study consisted of seven experiments.

Experiment 1: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on lychees cv. Kom flowering.

1.1 Experimental plants

The experiment was conducted by using 6 months old layering propagated lychees cv. Kom grown with fine sand in 37.5 cm (15 inch) diameter pots (Figure 2). The major elements for these trees provided in form of a concentrated solution, which composed of Mg^{2+}, K^{+}, Ca^{2+}, SO_4^{2-}, $H_2P0_4^{-}$ 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 every three days. The trees were grown in a fruit tree nursery at Department of Horticulture, Chiang Mai University, Thailand.

1.2 Experimental design

The factorial experiments in completely randomized design with seven replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on September 30, 2003. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at ten concentrations was applied to six months old lychees. The concentrations using in this experiment (g/l/pot) were

Treatment 1 = control (untreated)

Treatment 2 = $KClO_3$ and PP333 = 1 : 9 g/l/pot

Treatment 3 = $KClO_3$ and PP333 = 1 : 7 g/l/pot

Treatment 4 = $KClO_3$ and PP333 = 1 : 5 g/l/pot

Treatment 5 = $KClO_3$ and PP333 = 3 : 9 g/l/pot

Treatment 6 = $KClO_3$ and PP333 = 3 : 7 g/l/pot

Treatment 7 = $KClO_3$ and PP333 = 3 : 5 g/l/pot

Treatment 8 = $KClO_3$ and PP333 = 5 : 9 g/l/pot

Treatment 9 = $KClO_3$ and PP333 = 5 : 7 g/l/pot

Treatment 10 = $KClO_3$ and PP333 = 5 : 5 g/l/pot

Figure 2 Lychee cv. Kom grown in pots

1.3 Methods

After the trees were treated, the following data was recorded:

a. flowering time (days after treatment)
b. flowering percentage/tree (%)
c. number of panicle/tree
d. panicle width (cm)
e. panicle length (cm)

Experiment 2: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 2 years old lychees cv. Chakrapad flowering.

2.1 Experimental plants

The experiments were conducted by using 2 years old layering propagated lychees cv. Chakrapad grown with fine sand in 15 inches diameter pots. The major and minor nutrients elements for these trees provided the same as in experiment 1. The mixture of major and minor elements nutrient solution was given every three days. The trees were grown in the fruit tree nursery at Department of Horticulture, Chiang Mai University, Thailand (Figure 3).

2.2 Experimental design

The completely randomized design for four treatments with eight replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on July 2, 2004. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to two years old lychees. The concentrations using in this experiment (g/l/pot) were

Treatment 1 = control (untreated)
Treatment 2 = $KClO_3$ and PP333 = 1.00 : 9.00 g/l/pot
Treatment 3 = $KClO_3$ and PP333 = 1.25 : 8.75 g/l/pot
Treatment 4 = $KClO_3$ and PP333 = 1.67 : 8.33 g/l/pot

2.3 Methods

After the trees were treated, The following data was recorded:

a. flowering time (days after treatment)
b. flowering percentage/tree (%)
c. number of panicle/tree
d. panicle width (cm)
e. panicle length (cm)

Figure 3 Lychee trees cv. Chakrapad grown in pots

Experiment 3: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 8 years old lychees cv. Chakrapad flowering.

3.1 Experimental plants

The experiments were conducted by using 8 years old lychees cv.Chakrapad grew in the field condition at Chaing Rai Agricultural Research and Training Center, Chiang Rai Province, Thailand.

3.2 Experimental design

The completely randomized design for four treatments with four replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on July 4, 2004. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to eight years old lychees. The concentrations using in this experiment (g/tree) were

Treatment 1 = control (no chemical)

Treatment 2 = $KClO_3$ and PP333 = 70.0 : 630.0 g/tree

Treatment 3 = $KClO_3$ and PP333 = 87.5 : 612.5 g/tree

Treatment 4 = $KClO_3$ and PP333 = 117.0 : 583.0 g/tree

3.3 Methods

After the trees were treated, the following data was recorded:

a. flowering time (days after treatment)
b. number of flowering tree
c. flowering percentage/tree (%)
d. panicle length (cm)
e. panicle length (cm)

Experiment 4: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 2 years old lychees cv. Hong Huay off-season flowering at immature leaves stage.

4.1 Experimental plants

The experiments were conducted by using two years old lychees cv. Hong Huay grew in the field condition at Chiang Rai Agricultural Research and Training Center, Chiang Rai province, Thailand (Figure 4).

4.2 Experimental design

The completely randomized design for four treatments with four replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on July 4, 2004

when the leaves were at immature stage. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to two years old lychees. The concentrations using in this experiment (g/tree) were

Treatment 1 = control (untreated)

Treatment 2 = $KClO_3$ and PP333 = 20 : 180 g/tree

Treatment 3 = $KClO_3$ and PP333 = 25 : 175 g/tree

Treatment 4 = $KClO_3$ and PP333 = 33 : 167 g/tree

4.3 Methods

After the trees were treated, the following data was recorded:

a. flowering time (days after treatment)
b. flowering percentage (%)
c. number of panicle/tree
d. panicle width (cm)
e. panicle length (cm)

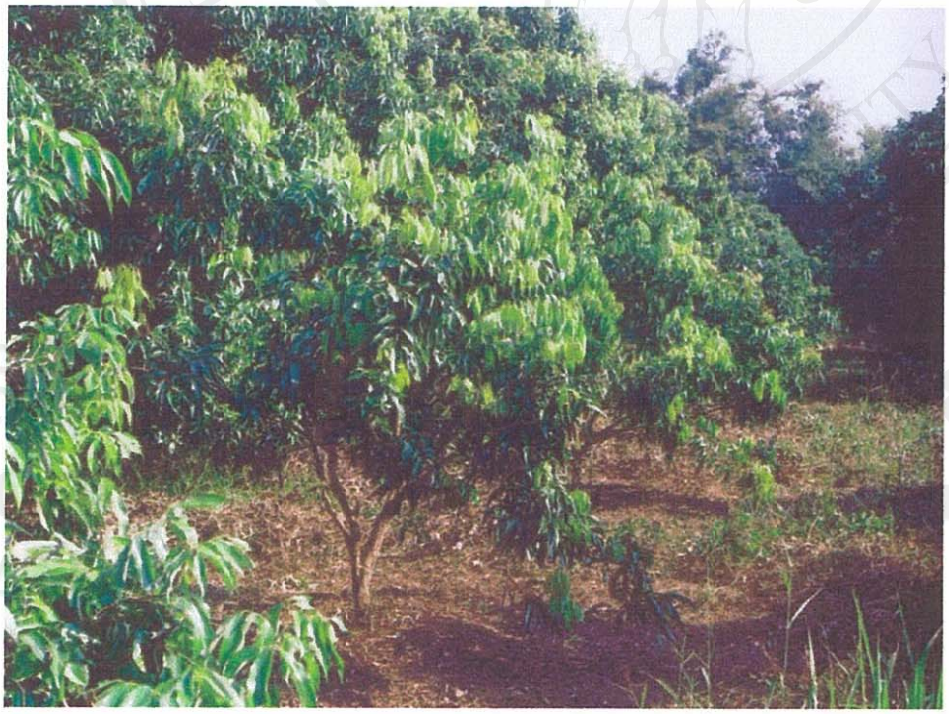

Figure 4 Canopy of 2 years old lychee trees cv. Hong Huay

Experiment 5: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 2 years old lychees cv. Hong Huay off-season flowering at mature leaves stage.

5.1 Experimental plants

The experiments were conducted by using two years old lychees cv. Hong Hauy grew in the field condition at Chiang Rai Agricultural Research and Training Center, Chiang Rai province, Thailand.

5.2 Experimental design

The completely randomized design for four treatments with four replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on July 4, 2004 when the leaves were at mature stage. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to two years old lychees. The concentrations using in this experiment (g/tree) were

Treatment 1 = control (untreated)

Treatment 2 = $KClO_3$ and PP333 = 20 : 180 g/tree

Treatment 3 = $KClO_3$ and PP333 = 25 : 175 g/tree

Treatment 4 = $KClO_3$ and PP333 = 33 : 167 g/tree

5.3 Methods

After the trees were treated, the following data was recorded

a. flowering time (days after treatment)
b. flowering percentage (%)
c. number of panicle/tree
d. panicle width (cm)
e. panicle length (cm)

Experiment 6: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 14 years old lychees cv. Hong Huay off-season flowering.

6.1 Experimental plants

The experiments were conducted by using 14 years old lychees cv. Hong Huay grew in the field condition at Chiang Rai Agricultural Research and Training Center, Chiang Rai province, Thailand (Figure 5).

6.2 Experimental design

The completely randomized design for four treatments with four replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on July 4, 2004 when the leaves were at mature stage. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to fourteen years old lychees. The concentrations using in this experiment (g/tree) were

Treatment 1 = control (untreated)

Treatment 2 = $KClO_3$ and PP333 = 100 : 900 g/tree

Treatment 3 = $KClO_3$ and PP333 = 125 : 875 g/tree

Treatment 4 = $KClO_3$ and PP333 = 167 : 833 g/tree

6.3 Methods

After the trees were treated, the following data was recorded:

a. time of leaf flushing
b. leaf flushing percentage (%)
c. number of new shoot/old shoot
d. leaf length (cm)
e. leaf width (cm)
f. flowering time (days after treatment)
g. flowering percentage/tree (%)
h. panicle length (cm)
i. panicle width (cm)

Figure 5 Canopy of 14 years old lychee trees cv. Hong Huay

Experiment 7: Effect of potassium chlorate ($KClO_3$) and paclobutrazol (PP333) on 14 years old lychees cv. Hong Huay on-season flowering.

7.1 Experimental plants

The experiments were conducted by using 14 years old lychees cv. Hong Huay grew in the field condition at Chiang Rai Agricultural Research and Training Center, Chiang Rai province, Thailand.

7.2 Experimental design

The completely randomized design for four treatments with four replications, one tree per replication, was employed in this experiment. Potassium chlorate with paclobutrazol was applied by soil drenching on November 16, 2004 when the leaves were at mature stage. Potassium chlorate ($KClO_3$) with paclobutrazol (PP333) at four concentrations was applied to fourteen years old lychees. The concentrations using in this experiment (g/tree) were

Treatment 1 = control (untreated)

Treatment 2 = $KClO_3$ and PP333 = 100 : 900 g/tree

Treatment 3 = $KClO_3$ and PP333 = 125 : 875 g/tree

Treatment 4 = $KClO_3$ and PP333 = 167 : 833 g/tree

7.3 Methods

After the trees were treated, the following data was recorded:

a. time of leaf flushing
b. leaf flushing percentage (%)
c. number of new shoot/old shoot
d. leaf length (cm)
e. leaf width (cm)
f. flowering time (days after treatment)
g. flowering percentage/tree (%)
h. panicle length (cm)
i. panicle width (cm)
j. auxin like-substances
k. gibberellin like-substances
l. cytokinin like-substances
m. ethylene
n. total non-structural carbohydrate (TNC)
o. reducing sugar (RS)

The chemical aspects

1. Indole-3-acetic acid purified were modified from Srikasetsarakul (2007). Two leaves of the terminal shoot were detached and placed with their petioles into the cavities of plastic plates. Each cavity was filled with 2.5 ml of 0.1 M phosphate buffer, pH 6.2 and incubated in darkness at 20° C for 20 hours. Thereafter the leaves were removed and the plates frozen and kept at -20°C until analysis. Liquidify the sample and add an internal standard (3-indolebutyric acid: IBA), 100ng/ml, 100µl. Shake well the solution and then add Polyvinylpyrrolidon: PVP approximately 10mg. Shake and leave it for 3 and 2 minutes accordingly. Centrifuge the sample at 4000

rpm for 10 minutes. Separate the dissolved solution to adjust pH at 2.5-3.0 with 4M acetic acid after that add diethyl ether as a half of the sample quantity. Shake and leave it for 2 and 5 minutes subsequently. The separated solutions can be observed. Bring the upper solution to evaporate, and then reprocess by adding diethyl ether again. Evaporate that solution under nitrogen gas atmosphere and dissolve by 50%methanol with 0.1M acetic acid, 200µl. Centrifuge the sample at 10,000 rpm at 15°C in the microcentrifuge for 15 minutes. Keep the sample in a tube to be analyzed by high performance liquid chromatography afterwards.

2. Gibberellin (GA) like-substances extraction and purification were modified from Nishijima *et al.* (1993). Twenty gram fresh weights of shoots were used for each analysis. Shoots were homogenized in a blender in 40 ml of 95% (v/v) n ethanol. 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 3°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 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 to 18 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 from PC.

Rice Micro-drop Bioassay (RMB), modified from Nishijima *et al.* (1993) was employed for GA bioassay. Taichung Native I, 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 ± 2^0 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 ± 2^0 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 quantity for GA from each sample by repeated the procedure again then compared with standard curve.

3. Ethylene was determined by modified from Kiatsakun (2004). Twenty grams of leaves was rapidly placed in the plastic container 3.7 litre. The gas was collected by syringe and quantifies by gas chromatography at 150°C injector, 120°C of oven, packed column, sieve-5A 80/100 and detector. Flow rate of nitrogen was 60 ml/mm and 50 k Pas for hydrogen and air. The concentration of ethylene was calculated and compared with standard ethylene.

4. Cytokinin-like substances extraction and purification were modified from Chen *et al.*, 1997. Twenty-five gram fresh weights of shoots were used for each analysis. Shoots were homogenized in a blender in 250 ml of 80% (v/v) ethanol. The mixture was kept at 4^0C 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 fraction 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 45^0C until it almost dry. Washed the evaporatory flask with 80% ethanol. About 1 ml of solution was collected for paper chromatogaphy. 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 microliter of the solution was applied as a 9-cm strip at 2 cm above the lower end of 9 x 28 cm Whatman 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 Rf l.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°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 (l963) 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.

5. Extraction of total non-structural carbohydrates (TNC) was using the acid extraction method described by Smith *et al.* (1964) and modified by Chaitrakulsup and Subhadrabandhu (1983)

Samples for chemical analysis;

1. Stem apex 15 cm. long from tip.

2. Fourth compound leaves counted from tip.

3. At the time when the lychee trees flowered or flushed leaves the new growth parts were cut off for supplementary chemical analysis, however the lower part, the stem apex 15 cm. measured from the junction of new and old growth parts

the fourth compound leaves counted from that junction were also taken for chemical analysis.

Sample preparation

After the samples were cut from the tree they were kept in the plastic bag and they were washed with distilled water and dried in the hot air oven at 60°C for 72 hours. Then the sample were ground in the wiley intermediate mill passing through 40 mesh sieves, and kept in the paper bags in the cool dry place for chemical analysis.

Sample analysis

Total nonstructural carbohydrates (TNC) analysis.

1. Extraction of total nonstructural carbohydrates (TNC)

The procedure was modified from Smith *et al.* (1964), samples of 0.4 gram were weighed in 250 ml, erlenmeyer flask, with 40 ml, 0.2 N.H_2SO_4 the flask was covered with aluminum foil, and incubated in the hot air oven at 100°C for one hour, After cooling to room temperature the samples were neutralized with sodium hydroxide solution, then diluted to 50 ml with distilled water and filtered through Whatman No.42 filter paper without protein removing. Then, they were kept in the plastic bottle and put in deep freeze refrigerator at -20^0 C for TNC determination.

2. TNC determination

The TNC were determined by analyzing for reducing power using the Shaffer-Somogyi Copper-Iodometric Titration method (AOAC, 1975). Glucose standard solution was prepared for standard curve. The results were expressed as miligram TNC (equivalent to glucose) per gram dry weight of sample. 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.

3. Time and places

3.1 Time

The duration of this study was from June 2002 to October 2005.

3.2 Places

1. Faculty of Agriculture, Chiang Mai University, Chiang Mai Province, Thailand.

2. Chiang Rai Agricultural Research and Training Center, Chiang Rai Province, Thailand.

3. Chiang Rai College of Agriculture and Technology, Chiang Rai Province, Thailand.