Chapter 3

Material and Methods

1. Materials

1.1 Experimental plants

The experiments were conducted by using three-years-old mango trees cv. Pim Sen Mun, Khiew Sawoey and Nam Dok Mai grafted on Kaew and Choke Anan rootstocks. The grafted mango trees were grown in the 50 cm diameter concrete pots, about 50 liters, using sand culture. The major elements for these trees provided in form of 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 elements. The minor elements used followed that advice of Hoagland and Arnon (1952). This solution had pH = 6.5. The mixture of major and minor elements nutrient solution was given every morning through out the experiments. All trees were grown in the experimental plot at the Department of Horticulture, Chiang Mai University (latitude 18° 47′ N, 312 m asl), Thailand during May 1998 to April 2000.

1.2 Other apparatus

1.) Electronics balance
2.) Oven and Desiccator
3.) Electronics Blender
4.) Titrator, Pipette and Micropipette
5.) Hand refractometer
6.) Spectrophotometer model Spectronics 21
7.) MacroKjeldahl Apparatus (Digestion unit model BuCHI 426 and Distillation unit model BuCHI 323) at Department of Biology, Faculty of Science, Chiang Mai University.
8.) Atomic absorption spectrophotometer (AAS) Varian model SpectrAA. 20 Plus at Department of Soil and Fertilizer, Faculty of Agricultural Production, Maejo University.
9.) Photosynthetic measurement apparatus LICOR model Li-6200

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10.) Pressure Chamber PMS Instrument Company model 1003 and xylem exudate keeper apparatus at Department of Agronomy, Faculty of Agricultural Production, Maejo University.

11.) Freeze-dried apparatus at Laboratory of Natural Products, Chulabhorn Research Institute, Faculty of Medicine, Chiang Mai University.

12.) Radio-immunoassay with polyclonal antibodies apparatus (combined of G4- glass in-filters; preconditioned column combination of polyvinyl pyrrolidone (PVP), DEAE-Sephadex A-25 and C_{18} Sep-Pak cartridge; and vacuum concentrator.) at Institut für Obst-,Gemüse- und Weinbau, Universität Hohenheim, Stuttgart, Germany.

13.) Silicone and hardener for Silicone impression.

14.) Microscope slide and acetate paper mixed with acetone solution.

15.) DAS Mikroskop LEICA DMLB (Brightfield) interface with LEICA Q 500 Image Processing and Analysis system at Pathology division, Regional Medical Sciences Center, Chiangmai.

16.) Stop watch and Infiltrated on leaves apparatus

17.) Growth Chamber

18.) Rice var. Phrea 1 for Rice Secondary Leaf Sheath Bioassay (RSLSB)

19.) Paper chromatogram , Chamber, capillary tubes and alcoholic burner.

20.) Vernier caliper, ruler and Length-measuring-tape

21.) Freezer (controlled temperature at-20°C and –70°C)

22.) Muffle Furnace and porcelain crucible

23.) Meteorological data of Northern-Region Meteorological Center, Chiang Mai for the period between May 1998 to April 2000

2. Experimental Design

A 2x3 Factorial in Completely Randomized Design (CRD) was employed. There were 10 replications in each treatment with one mango tree represented one replicate. While factor A was 2 rootstocks cv. Kaew and Choke Anan, whereas factor B was 3 scions cv. Pim Sen Mun, Khiew Sawoey and Nam Dok Mai (Checked with Choke Anan grafted on Kaew rootstock was used as extra comparison not involved in the experimental design nor statistical calculation)

Methods

Collected the data;-

1.) **The growth and development of mango trees** (Manochai, 1992)

a. **Stem height** measured from the mark at the edge of pot to the highest point of the shoot in centimeter.

b. **Canopy width** measured from the widest of canopy width (marked at the edge of pot, from East to West and North to South, calculated the mean, measured in centimeter.

c. **Stem diameter** measured at 2 cm higher than grafted level of mango trees, marked the position on them for further measurement, used Vernier Caliper, measured in centimeter.

The data were collected monthly and were used to calculate the growth rate of the trees with the following Empirical formula :

$$R = \frac{X_t - X_0}{X_0} \times 100\%$$

R = the percentage of growth rate

X_t = the last measured data

X_0 = the first measured data

2.) **The growth and development of terminal shoots** (Manochai, 1992)

a. **Types and Numbers of emerging shoots and inflorescenses** when they were fully grown, collected the number of inflorescences and new shoots.

b. **Number and percentage of shooting and flowering** , by counting the numbers and labeling when the shoots and inflorescence were 2 cm long.

c. **Number of leaves per new shoots,** when the leaves were fully developed and expand.

d. **The length of new shoots** , when new shoot was 60 days old, measured from the junction between the new and the previous shoot to the tip of the new shoots.

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e. **Diameter of the new shoots,** measured when they were green, measured 2 cm above the junction between the new and old terminal shoots.

f. **The width and the length of the new leaves,** chose the 3rd to 5th leaves from the base of new shoots, measured the length and width of the mature leaves.

3.) **Analysis of fruit products and yields** (Changjeraja, 1996)

a. **Ratio of male to perfect flower** randomly selected the inflorescences and counted the number of flower at 80% full bloom.

b. **Percentage of fruit-setting,** randomly by selected the inflorescences and counted the number of fruit 2 times when the fruits were match's head, and 1.5 cm diameter ; then calculating against the number of perfect flowers in that inflorescence.

c. **Average numbers of fruits per tree,** counted all the mature fruits.

4.) **The qualities of fruits,** physical and chemical properties were collected when the mango fruits were mature* as the following data:

a. **The weight of fresh fruits,** weighed the fruits in gram.

b. **The size of fruits,** measured the widest side of fruit as the width; the length measured from the base to the tip of the fruit, and the thickness in centimeter.

c. **The amount of titratable acid: TA,** diluted 5 ml of mango's fruit juice with distilled water to 25 ml, then titrated with 0.1N NaOH using 1% Phenolpthalene as indicator (Rujjanakrikant and Rattanapanont, 1990). Calculated TA as citric acid equivalent as the empirical formula;

$$TA = \frac{\text{volume of NaOH x concentration of NaOH x mol.wt.of citric acid x100\%}}{\text{Volume(ml) of fruit juice}}$$

d. **Total soluble solids : TSS;** measured by using hand refractometer from mango's juice, in °Brix. (Rujjanakrikant and Rattanapanont, 1990)

5.) **Measurement of net photosynthetic rate;** used Li-6200 Portable Photosynthesis system (Li-COR.Inc) measured the 3rd to 5th 8 months old perfect intact leaves from the terminal shoot, in $\mu mol\ CO_2\ m^{-2}s^{-1}$(Phattaralerphong, 1997).

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* Pim Sen Mun harvested at about 95 days after Inflorescence emerged, Khiew Sawoey and Nam Dok Mai harvested at about 91 and 100 days after 100% full bloom, respectively (Panthukhasemsuk, 1990), and Choke Anan harvested at about 110 days after 80% full bloom (Sawanichawong *et al.*, 1991)

6.) Analysis of chlorophyll a and b content in the leaves by Whitham *et al.,* (1971) method; measured the optical density of the sample by spectrophotometer at wave- length 645 and 663 nanometers, calculated and reported in milligrams of chlorophyll per gram fresh weight of leaves.

7.) Stomatal behavior

a. **Stomatal width, length and density;** used visual observation by silicone rubber impression method (Tunsuwan and Bünemann, 1973) using the center of the lower surface of the third leaf from the apex of terminal shoots of the well-watered mango trees (used the same position in the same leaves throughout the experiment). Measured trees hourly between the period of 06.30 a.m. to 18.30 p.m.. Printed the surface of silicone rubber replicas on microscope slides by flooding the slides surface with the mixture of acetate paper and acetone solution, then pressed the silicone rubber replicas on the slide. This was then allowed to dry and resulted in negative replica of the surface silicone rubber replicas, that mean—the positive replica of the topographic features of the leaf surface on microscope slides. Finally, study the stomatal behavior under DAS mikroskop LEICA DMLB (Bright field) interface with LEICA Q 500 Image Processing and Analysis system; measured the width and the length of stomata in unit : micron (μm) ; and randomly counted stomatal density (by using lymphocyte area random counted) in unit : numbers per mm^2.

b. **The infiltration rate of leaves;** applied a 2 mm diameter-spot of 1:1 mixture solution of petroleum benzine (octane number 91) and Isopropanol on the center of the third the lower surface of leaf from the apex of terminal shoots (used the same position of leaves but not the same leaves) hourly from 06.30 a.m. to 18.30 p.m. Measured the time that these mixed solvent infiltrated the leaves (Tunsuwan, 1976) in unit : second (s).

8.) Dry weight of roots, stems, leaves and whole plants; for the leaves, kept at 72°C for 48 hours in an oven, while the stems and roots were sun dry for 15-21 days, then oven dry at 72°C for 48 hours. Weighed these dry matter on Electronic balance (precision 2 decades).

9.) Study on carbohydrates, mineral elements and endogenous hormones in the leaves, terminal shoots and xylem exudate, forty terminal shoots centrally located around the canopy of similar size and with uniform bud development were collected from each of the experimental trees during inflorescence development in 4 stages (shown in Figure 3.1) as followed:

stage 1 = mature terminal shoots (ready for bud-break)

stage 2 = bud-break (bud emergence with whitish top)

stage 3*= bud emergence, Inflorescence 3-4 cm long

stage 4*= bud emergence, Inflorescence 10-12 cm long

(* in 3rd and 4th stage, the inflorescence were cut off remained only the terminal shoots for analysis)

Figure 3.1 The 4 stages of inflorescence development which collected from each of the experimental tree

The length of the terminal shoots were 5 cm long from the apex of shoots. Collected 6 leaves from each terminal shoots. They were of regular, similar and matured at the 3rd to 5th leaf position from the apex of shoot. The leaves sample were divided into in two parts: first, kept for

analysis of carbohydrate and nutrients by cleaning with distilled water and blot dry with clean tissue paper. Kept the samples in the sealed paper bags and oven dry at 70 °C for 72 hours. Then grind to powder with blender and kept them in desiccator. Before analysis of carbohydrate and nutrients, oven dry again at 70°C for 8 hours.

Another part of leaves and terminal shoot sample were kept for GA-like substances analysis. The terminal shoots were sealed in plastic bags and quickly placed in a chilled, insulated container and taken to the laboratory, cleaned them with distilled water and blot dry between tissue paper, stored in a deep freezer at -70°C for later extraction and determination of GA-like substances.

a. Analysis of carbohydrates

1. Extraction of total nonstructural carbohydrates (TNC). The samples were extracted by using the acid extraction method described by Smith *et al.* (1964) and modified by Chaitrakoolsup and Subhadrabandhu (1983).

2. Extraction of reducing sugars. The samples were extracted by using the ethanolic extraction method described by Yemm (1935).

3. Determination of carbohydrates. The determination of total nonstructural carbohydrates(TNC) and reducing sugars (RS) was done using Nelson's reducing sugar procedures (Hodge and Hofreiter, 1962), expressing the amount of carbohydrates as mg D-glucose equivalent.

b. Analysis of mineral elements

1. **Analysis of total nitrogen (TN)** by Kjeldahl methods (Attanant *et al.,* 1989).
2. **Analysis of phosphorous** by Vanadomolybdate methods (Attanant *et al.,* 1989).
3. **Analysis of potassium, calcium, magnesium** by Atomic absorption methods (A.O.A.C., 1990).

c. Analysis of GA-like substances.

GA-like substances were extracted from fresh terminal shoots under low temperature by 80% methanol and purified by paper-chromatography using the method described by Rahman *et al.* (1975) and Yokato *et al.*(1980) which modified to used in mango by Juthamanee (1989). GA-like substances were analyzed by Rice Secondary Leaf Sheat Bioassay (RSLSB) of rice variety Phrae 1 as described by Yopp *et al.* (1986) and modified by Phivnil (1999).

d. Analysis of cytokinins in xylem exudate.

1. **Collected xylem exudate,** used one year old mango trees cv. Kaew and Choke Anan about 8-10 millimeters in diameter planted at the Department of Horticulture, Chiang Mai University, Chiang Mai. Collected the xylem exudate by using pressure chamber and kept in silicone tube (Kamboj *et al.* 1999). This process was done at the Department of Agronomy, Maejo University, Chiang Mai. Xylem exudate was collected on January 10th –25th , 2000 at predawn (about 05.00 to 07.30 a.m.),the exudate was immediately frozen in salt mixed ice about –32°C and then quickly stored in a freezer at –20°C. Freeze-dried approimately 1 ml of the frozen exudate then stored at -20°C until extracted.

2. **Determination of zeatin/zeatin riboside (Z/ZR) and N^6-(δ^2-isopentenyl)adenine/ N^6-(δ^2-isopentenyl)adenosine(iP/iPA).**

   2.1 Hormone extraction, freeze-dried samples were ground in liquid nitrogen, homogenized and then extracted overnight with 50 ml 80% chilled aqueous methanol (below 0°C) in darkness at 4°C. The extracts were then filtered through G4-glassinter-filters (max. pore size 10-16 µm), dried under low temperature and dissolved in 12 ml 0.1 M ammonium acetate (pH 9.0) by using ultrasonic bath and subsequently frozen at -20°C overnight. After thawing, the extract was centrifuged at 22,000 rpm at 4°C for 25 min (Jimenez 2000).

   2.2 Purification, the supernatant from the centrifugation step was passed through a preconditioned column combination of polyvinylpyrrolidone (PVP; Sigma Chemical Co., Deisenhofen, Germany), DEAE-Sephadex A-25 (Pharmacia, Freiburg, Germany) and a C_{18} Sep-Pak cartridge (reversed phase, Waters, Eschborn, Germany) (modified from Bertling and Bangerth, 1995).

   2.3 Quantification of hormones, hormones were quantified by radio-immunoassay (RIA) with polyclonal antibodies. Antibodies used were raised in rabbits against free ZR and iPA. Radioimmunlogical hormone quatification was performed according to Bohner and Bangerth (1988).

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