CHAPTER 3
MATERIALS AND METHODS

3.1 Effect of High Carbon dioxide Pressure Treatments on Postharvest Quality in Longan Fruit

Mature uniform longan fruit were harvested in August 2008 from a GAP certified in Chiang Mai province. The fruit stems were cut-off, approximately 0.5 cm left. The fruit were treated with carbon dioxide at the pressures of 1, 1.5 and 2 kg-cm^{-2} for 1, 2 and 3 hr in the pressure tank. The high carbon dioxide pressures were obtained by increasing the atmospheric pressure with pure carbon dioxide from a carbon dioxide tank then the fruit were kept in a foam tray wrapped with PVC plastic and stored at 10 °C with 85 % relative humidity. The experimental design was a factorial with three replications of each pressure level. Longan fruit were treated with 1.0, 1.5 and 2.0 kg-cm^{-2} (3 levels) for 3 periods of time (1, 2 and 3 hours) of each carbon dioxide pressure.

3.1.1. Determination of pericarp and aril color

3.1.1.1. Browning Index

Longan samples, fifteen fruit per replication were evaluated with a 5-score scale of pericarp browning (Figure 1). Browning scale was assessed by measuring total browning areas of the pericarp on each fruit as followed.

1 = no browning (excellent quality)

2 = less than 10% of surface

3 = less than 25% of surface

4 = less than 50% of surface

5 = more than 50% of surface

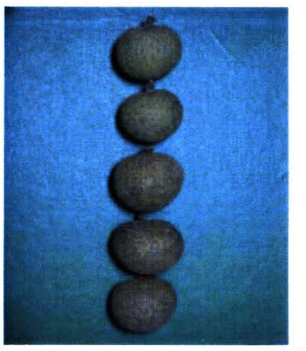

Figure 1 Browning scale of longan pericarp

Then browning indices were calculated using the following formula: Browning index = (browning scale x percentage of corresponding fruit within each class). Fruit having a browning index above 3.0 were rated as unacceptable (Jiang and Li, 2001).

3.1.1.2. Determination of pericarp color (outer and inner side) and aril color by using a colorimeter

The color of pericarp and aril were measured with a colorimeter (Color Quest XE) according to the CIELAB scale (Figure 2). The degree of browning was expressed as L*, Chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$) and hue angle ($h^o = \tan^{-1} b^*/a^*$) values. The results were expressed as a mean value from four replications of the 10 measured samples per replication.

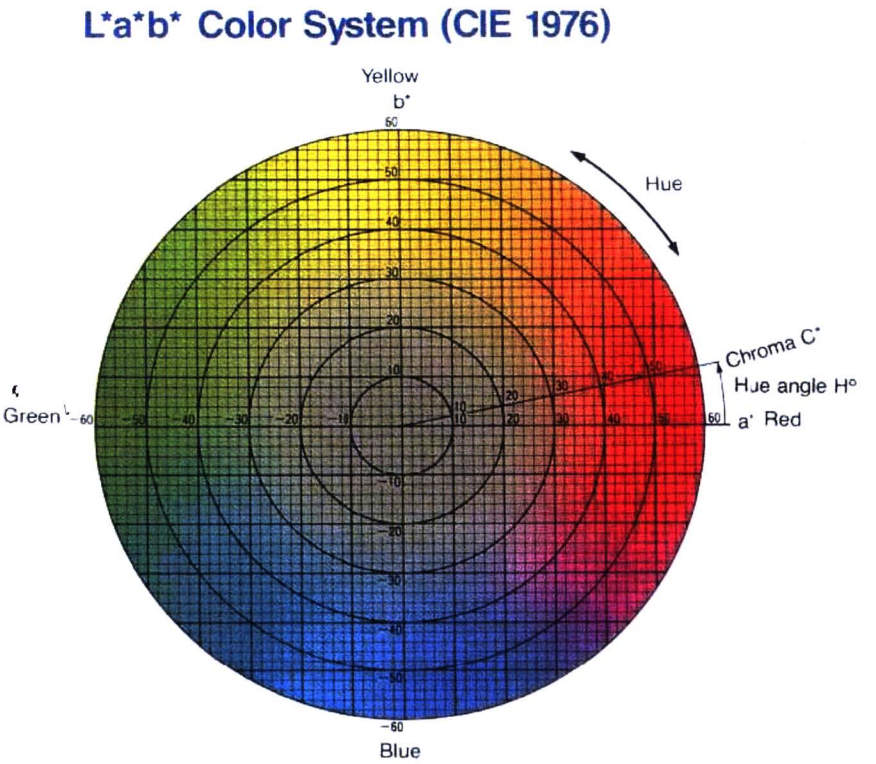

Figure 2 Color chart of Minolta model CR-300 (Minolta, 1976)

3.1.2. Determination of weight loss.

Weight loss percentage was also determined by weighing the whole fruit packed in foam tray before and after the storage. Then, it was calculated to percent by the following formula:

$$\text{Weight loss (\%)} = \frac{\text{weight before storage} - \text{weight after storage}}{\text{weight before storage}} \times 100$$

3.1.3. Determination of respiration rate

Longan samples (ten fruit per replication) were sealed in a 1 litter airtight glass jar for 1 hr, with three replications. Headspace gas sample, 1 ml was withdrawn with a gas-tight hypodermic syringe and analyzed by gas chromatography fitted with a 80/100 mesh Porapack-Q column and a thermal conductivity detector (CHROMATOPAC C-R 8A, SHIMADSU Co., Kyoto, Japan). While respiratory rate was expressed as mg $CO_2.hr^{-1}.kg^{-1}$ (fresh weight)

3.1.4 Determination of ethylene production

Three replicates of each ten fruit sample were used to determine rates of ethylene production. Fruit of similar size was sealed in each 1 L jar for 1 hour at room temperature (25°C). Gas samples of 1 ml were withdrawn from each jar and the concentration of ethylene determined using a GC14-B Shimadzu gas chromatograph equipped with an activated aluminium column and a flam ionization detector (FID) detector. Temperature was set at 150, 55 and 150 °C for injector, oven and detector, respectively. Helium was used as a gas carrier (1.25 kg/cm).

3.1.5 Evaluation of disease incidence and overall quality acceptance

Disease incidence percentage was visually observed on a number of the fruit that showed lesions of mycelium or rot on the fruit surface area.

Overall quality acceptance of outer pericarp, inner pericarp and aril were evaluated by scoring each from 1-5. Five well-trained evaluators scored each fruit as 1 = most dislike, 2 = moderately dislike, 3 = neither like nor dislike, 4 = moderately like, and 5 = most like. Panelists were given one fruit per treatment.

3.2. Effect of High Carbon dioxide Pressure Treatments and Storage Temperature on Some Chemical Components and Biochemical Characteristics in Longan Fruit

Mature uniform longan fruit were harvested in August 2008 from an orchard in Chiang Mai province. The fruit stems were cut-off, approximately 0.5 cm left. The fruit were treated with carbon dioxide at the pressures of 2.0 kg-cm^{-2} for 1, 2 h and untreated (control) in the pressure tank. The high carbon dioxide pressures were obtained by increasing the atmospheric pressure with pure carbon dioxide from a carbon dioxide tank then the fruit were kept in a foam tray wrapped with PVC plastic and stored at 5 and 10 °C with 85 % relative humidity. The experimental design was a factorial (3 carbon dioxide treatment x 2 storage temperature) with four replications of each treatment.

3.2.1. Effect of High Carbon dioxide Pressure Treatments and Storage Temperature on Some chemical Components in Longan Fruit

3.2.1.1 Measurement of pH and titratable acidity (TA) of aril and pericarp

Ground sample (10g) was homogenized with 10 ml distilled water. The pH was measured with pH meter (Consort 431, Belgium). Titratable acidity was determined. Sample was titrated with 0.1 N NaOH solutions to pH 8.2 using autotitrator (Titroline easy, Schott, Mainz, Germany) under continuous stirring and expressed as mg acid per 100 ml of pulp juice.

3.2.1.2 Determination of aril reducing sugars

Reducing sugars were measured according to the modified method of Nelson (1944). Ground aril sample (1g) was homogenized with 20 ml distilled water. 0.5 ml of distilled water and 1 ml of 1N sulphuric acid were added and incubated at 49 °C for 30 minutes. The solution was neutralized with 1 N sodium hydroxide using

methyl red indicator. One ml of Nelson's reagent was added to each test tube prepared by mixing reagent A and reagent B in 25:1 ratio (Reagent A: 25 g sodium carbonate, 25 g sodium potassium tartarate, 20 g sodium bicarbonate and 200 g anhydrous sodium sulphate in 100 ml: Reagent B: 15 g cupric sulphate in 100 ml of distilled water with 2 drops of concentrated sulphuric acid). The test tubes were heated for 20 minute in a boiling water bath, cooled and 1 ml of arsenomolybdate reagent (25 g ammonium molybdate, 21 ml concentration sulphuric acid, 5 g sodium arsenate dissolved in 475 ml of distilled water and incubated at 37 °C in a water bath for 24 hours) was added. The solution was thoroughly mixed and diluted to 25 ml and measured at 540 nm in a spectrophotometer. The reducing sugars content of sample were calculated from glucose standard. It was calculated to percentage of reducing sugars.

$$\text{Reducing sugars (\%)(as sucrose)} = \frac{\text{mg sucrose} \times 200\text{ ml} \times 100}{\text{Sample Wt.(g)} \times 20\text{ ml} \times 1000\text{mg/g}}$$

3.2.2 Effect of High carbon dioxide pressure treatments and storage temperature on some biochemical characteristics in longan fruit

3.2.2.1. Determination of respiration rate

Longan samples fifteen fruit per replication were sealed in a 1 litter airtight glass jar for 1 hr, with four replication. Headspace gas sample, 1 ml was withdrawn with a gas-tight hypodermic syringe and analyzed by gas chromatography fitted with a 80/100 mesh Porapack-Q column and a thermal conductivity detector (CHROMATOPAC C-R 8A, SHIMADSU Co., Kyoto, Japan). While respiratory rate was expressed as mg $CO_2 hr^{-1} Kg^{-1}$(fresh weight)

3.2.2.2 Extraction and analysis of phosphofructokinase (PFK), pyrophosphate :fru-6-P phosphotransferase (PFP) and pyruvate kinase (Nanos *et al.*,1994)

Extraction and analysis of phosphofructokinase (PFK)

Extraction

These two enzymes were extracted and assayed via a modification of the method of Smyth *et al.* (1984). Fruit pericarp were ground to a fine powder under liquid N. Four grams of powder were extracted in 8 ml medium containing 100 mM tris-HCl (pH 8.0), 2 mM disodium-EDTA, 1 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 200 mg insoluble (40,000 molecular weight) polyvinylpyrollidone (PVP). The solution was centrifuged at 20,000 x *g* for 25 min to removed debris, and the resulting supernatant (crude homogenate) was used for enzyme assay.

The reaction medium for PFK: (total volume 2 ml) contained 90 mM hepes-NaOH buffer (pH 8.0), 2.5 mM MgCl2, 10 mM fru-6-Pm, 0.16 mM NADH, 1.2 units aldolase, 1.8 units glycerol-1-P dehydrogenase, 14 units triosephosphate isomerase, and 200 ml supernatant. The reaction was initiated by adding 1 mM sodium-ATP. The absorbance at 340 nm was measured for at least 5 min by using a spectrophotometer. During the impletation the temperature was maintained at 25 °C for keeping the rate of enzyme activity. The activity is expressed in mmoles NADH oxidized/mg protein per min.

Analysis of pyrophosphate: fru-6-P phosphotransferase (PFP)

The reaction medium for PEP: (total volume 2 ml) contained 90 mM hepes-NaOH buffer (pH 8.0), 2.5 mM MgCl2, 10 mM fru-6-Pm, 0.16 mM NADH, 1.2 units aldolase, 1.8 units glycerol-1-P dehydrogenase, 14 units triosephosphate isomerase, and 200 ml supernatant. The reaction was initiated by adding 1 mM sodium-pyrophosphate and 2 mM fructose- 2, 6 P2. The absorbance at 340 nm was measured for at least 5 min by using a spectrophotometer (model PM2-DL; Zeiss, New York). During the impletation the temperature was maintained at 25 °C for keeping the rate of enzyme activity. The activity is expressed in change in absorbance/mg protein per min.

Extraction and analysis of pyruvate kinase (PK)

Five grams of tissue from the equatorial area, including pericarp, was homogenized in 20 ml extraction buffer (100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.5, 5 mM DTT, and 0.5% soluble PVP for 20 sec using a homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000 x *g* for 10 min. The supernatant was immediately used for enzyme assay. The PK assay mixture were contained 50 mM MES buffer (pH 6.0), 2.5 mM ADP, 1.5 mM PEP, 4 mM MgCl2, 25 mM KCl, 0.1 mM NADH, and 3 units of lactate dehydrogenase. The reaction was initiated by adding 100 ml supernatant (total volume 1 ml), and the decrease in absorbance at 340 nm was followed at 25 °C. The rate was linear for at least 3 min, and the activity was reported as mmoles NADH oxidized/mg protein per min.

3.2.2.3. Determination of ethylene production

For each sample, four replicates of ten fruit each, were used to determine rates of ethylene production. Fruit of similar size were sealed in 1 L jars for 1 hour at room temperature (25 °C). Gas samples of 1mL were withdrawn from each jar and the concentrations of ethylene determined using a gascromatography (GC) 14-B Shimadzu gas chromatograph equipped with an activated aluminium column and a FID detector. Temperatures were 150, 55 and 150 °C for injector, oven and detector, respectively. Helium was used as a gas carrier at 1.25 kg/cm.

3.2.2.4. Analysis of ACC synthase, ACC oxidase activity, and ACC concentration (Kondo *et al.*, 2006)

ACC extraction and analysis was estimated according to Kondo *et al.* (1991). Analysis of ACC synthase wasperformed with a modification by Jiang *et al.* (1994). Samples were homogenized with 3ml/g of ice-cold extraction buffer [50mM Tris–HCl buffer containing 1mM dithiothreitol (DTT) and 1mM phenylmethylsulfonyl fluoride (PMSF) at pH 8.2]. The homogenate wascentrifuged at 10,000 x g for 20 min at 1°C. The supernatant was desalted through a Sephadex G25 column (PD-10). For determination of the activity of ACC synthase, 0.9 ml of desalted extract was placed in a glass tube with 50mM SAM, 4mM pyridoxal phosphate, and 50mM Tris–HCl

buffer containing 1mM DTT and 1mM PMSF at pH 8.2 of 0.1 ml. For analysis of ACC oxidase, each excised flesh sample of 2 g was dipped into 15 ml of 0.4M mannitol and 10mM phosphate buffer, containing 5mM ACC in 50 ml flask. After the ethylene in the sample was removed by low pressure for 5 min, the flask was closed and then was placed at 25 C for 2 h. Ethylenne in the head space of the syringe was determined by GC -14B.

3.2.2.5 Determination of polygalacturonase (PG) activity in the pericarp

Extraction of polygalacturonase

PG was extracted according to the method of Peng (2004) with some modifications. Three grams of ground pericarp sample were homogenized in 10 ml of 0.1 M phosphate buffer at pH 6.4 containing 0.1 M ascorbic acid , 5% insoluble polyvinylpyrrolidone(PVPP), and 1 mM ethylenediamine tetraacetic acid(EDTA). Then homogenate was filtered through two layers of cheesecloth and centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was collected and kept at -80 °C for PG assay.

Polygalacturonase assay

PG activity was determined by measuring the reducing groups released from polygalacturonic acid. Reducing groups were measured with 3, 5 dinitrosalicylic acid with slight modification of method described by Peng (2004). The reaction mixture, containing 1 ml of 0.5 % polygalacturonic acid, 1 ml of 0.1 M sodium acetate buffer (pH 6.4) were incubated at 30±2 °C for 50 min. The samples were taken every 10 min to added 1 ml of 3,5 dinitrosalicylic acid solution and boiled at 95±3 °C for 10 min. The absorbance was recorded at wavelength 575 nm. PG was expressed in absorbance per min per mg protein (OD_{575} min^{-1} $protein^{-1}$).

3.2.2.6 Determination of Polyphenol oxidase (PPO) enzyme activity (Su *et al.*, 2005)

The pericarp from ten fruit of each treatment were frozen with liquid nitrogen and then powdered using a blender. The powdered pericarp (3.0 g) was homogenized in 24 ml of 0.1 M phosphate buffer (pH 6.4) at 4°C. The homogenate was centrifuged at 15,000 x g (Herolab-Unicen 15 DR, Germany) for 20 min and then the supernatant was collected to assay PPO activity according to the modified method of Jiang (1999), by measuring the oxidation of pyrocatechol. The increasing in absorbance capacity at 400 nm at 25°C was automatically recorded for 5 min, using a spectrophotometer (SPE Cord M 40, Germany). One unit of enzyme activity was defined as the amount causing a change of 0.001 in absorbance capacity per minute. The protein content was determined according to the dye-binding method of Bradford (1976) using albumin bovine serum as the standard.

3.2.2.7 Evaluation of disease incidence

Disease incidence percentage was visually observed on a number of the fruit that showed lesions of mycelium or rot on the fruit surface area.

3.3 Effect of High Carbon dioxide Pressure on Inoculated Longan Fruit and Mycelium Growth of *Pestalotiopsis* sp.

The longan fruit were inoculated with 0.5 diameter mycelium disc placed on the stem-end of fruit and inoculated at 25 °C for 12 hours. The non inoculated fruit and *Pestalotiopsis* sp.- inoculated fruit were treated with carbon dioxide at the pressures of 2.0 kg-cm^{-2} for 1, 2 hr and untreated in the pressure tank. The high carbon dioxide pressures were obtained by increasing the atmospheric pressure with pure carbon dioxide from a carbon dioxide tank. After the treatments, inoculated fruit were kept at ambient temperature until they showed significant mold. The fruit decay was assessed by visual appearance scale as described in experiment 3.1

Mycelium growth of *Pestalotiopsis* sp. was measured: *Pestalotiopsis* sp. was cultured on PDA for 3 days. Using a sterile cork borer 4 mm mycelial plugs was aseptically transferred to the center of fresh PDA. Five replications were treated

with the carbon dioxide at the pressures of 2.0 kg-cm^{-2} for 1, 2 hr and untreated (control) in the pressure tank. The high carbon dioxide pressures were obtained by increasing the atmospheric pressure with pure carbon dioxide from a carbon dioxide tank. Mycelium growth (radial colony diameter) was measured after 3, 5 and 7 days. Morphology of fungus was investigated at interval during high pressure treated compared with untreated under compound microscope.