CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Plant materials

The plant material used in this research is Pak-choi or Pak Kad Hong Tae in the Thai language, the variety is 'Brisk Green' from the Known-You Seed (Thailand) Co., Ltd. Pak-choi samples were grown under the organic standards of ACT (IFOAM Accredited). They were cultivated in a farmer's greenhouse in Muang Ang village, Chom Thong district, Chiang Mai province, Thailand. Fifteen-day pak-choi seedlings were transplanted. Then 24-27 days after transplanting, organic pak-choi were harvested from the planting plot. Then they were brought to the Royal Project packing house (the produce were transported immediately after harvesting), located about 87 km from the farmer's plot in Muang district, Chiang Mai province. The vegetable was sorted for uniformity, trimmed, and packed in 25×40 cm perforated polyethylene bags with 18, 0.8 cm diameter holes. Each bag contained 300 g pak-choi samples. Postharvest handling of pak-choi from farm to the Royal Project Produce Center was show in Figure 3.1.

Figure 3.1 Postharvest handling of pak-choi from farm to the Royal Project Produce Center, Chiang Mai

3.1.2 Apparatus

Standard laboratory apparatus have been employed along with, in particular, the following:

1) Data logger, Hobo U12-012, Onset Computer Corporation, USA
2) SPAD 502 Plus, Konica Minolta Sensing, Inc., Japan
3) Infrared thermometer, Smartsensor, Model AR842A+, Oyeah Company, China
4) Analytical balance 2 decimal, maximum 600 g, BA3100P, Sartorius basic Co., Ltd., Germany
5) Analytical balance 2 decimal, maximum 600 g, EK-610i, AND Co., Ltd., Japan
6) Analytical balance 4 decimal, maximum 210 g, HR-200, AND Co., Ltd., Japan
7) Analytical balance 4 decimal, maximum 210 g, METTLER TOLEDO, Model: AB204, Switzerland
8) HPLC (LC-10AT pump, CTO-10A column oven, SCL-10A VP system controller, and SPD-M10A VP Diode Array Detector), Shimadzu, Japan
9) GC series 750 flame ionization detector, GOW-MAC Instrument CO., U.S.A.
10) Gas analyzer, Bridge Analyzers, Model 900151, USA
11) Hydro vacuum cooler, Hussmann Co., Ltd., USA
12) Spectophotometer, Genesys 20, Themo Scientific Co., Ltd., USA
13) Refrigerator, SBC-3D, SANYO
14) Freeze-dried, Dura-Stop, FTS System, USA
15) Water bath, WNB14, Memmert, Germany
16) Hot air oven, UNB 400, Memmert, Germany
17) Blender, Philips HR2068, Indonesia
18) Micropipette, 1,000 µl and 200 µl, Gilson, France
19) Centrifuge, Model 6930, Kubota, JAPAN
20) Furnace, Model: CARBOLITE, Omron, England
21) Desiccator, Boromax, USA
22) Crucible, HTC, Germany
23) Fiber bag, C. Gerhardt GMBH & CO. KG, Germany
24) Filter paper, Whatman N0.1
25) Micropipette, Model: Pipetman, GILSON, France

26) Glassware; Erlenmeyer flask, beaker, volumetric flask, cylinder, burette, pipette, etc.

27) Flow board for measuring respiration rate (Figure 3.1) include:

(1) Panel and wooden base

(2) Air in tube

(3) Glass tube for ventilation

(4) Glass cylinder with water

(5) Glass bottle with water

(6) Glass tube showing pressure level

(7) Capillary tube

(8) Gas tube

(9) Plastic box for storing the produce

(10) Air out tube

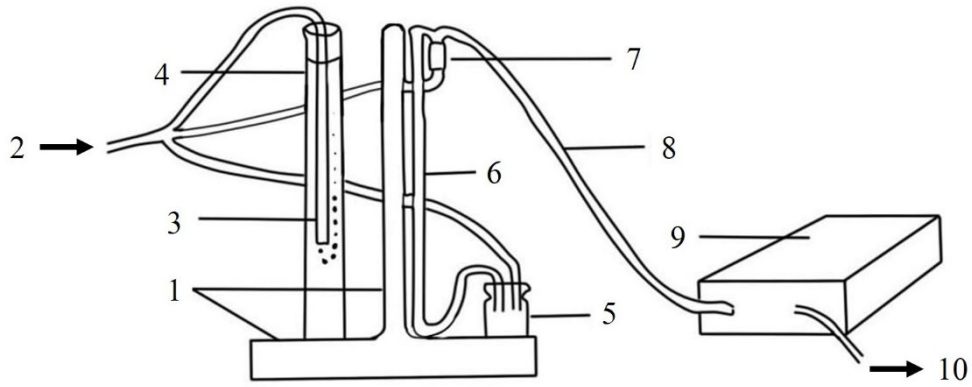

Figure 3.2 Flow board for measuring respiration rate

The principles of flow board are the air from air pump flow through air in (2) the air separates for 3 ways; flow into glass tube for ventilation (3), flow into glass bottle with water (5), and capillary tube (7). Then flow into plastic box which the produce was kept (9). In case of low air pressure, the air will flow through capillary tube because it cannot press the water in glass tube for ventilation (3) or in glass bottle (5). When air pressure rises the air will push the water in glass tube for ventilation (3) was lower and push the water in glass bottle (5) up to glass tube showing pressure level (6) which the level of

water was high as air pressure. If the air pressure increases, the water in the glass tube (3) was lowered to the bubbles at the end of the glass tube (3).

28) Air flow meter (Figure 3.2) include:

(1) Air in

(2) Burette

(3) Rubber ball with soap water

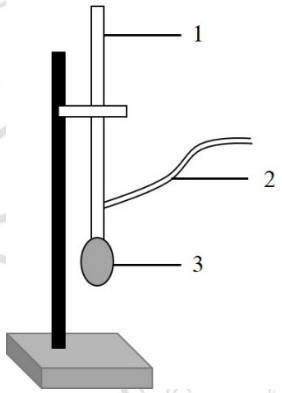

Figure 3.3 Air flow meter

The principle is to connect the air hose through the capillary tube in the flow board to the air flow meter. When the rubber ball is squeezed, the soapy water flows up and off the air. As air flows out of the capillary tube into the burette the air will push the soap bubbles out of the burette. Measure the flow rate of the air by measuring the movement of the bubbles then calculate the air flow rate in ml/minute.

3.1.3 Chemicals and preparation

1) The chemicals for vitamin C analysis
   (1) 0.4% oxalic acid, 4.0 g of oxalic acid (UNIVAR) were dissolved in distilled water and made up to 1,000 ml
   (2) 0.04% 2,6-dichlorophenol indophenols, 0.4 g of 2,6-dichlorophenol indophenols (SIGMA) was dissolved in distilled water and made up to 1,000 ml
   (3) Ascorbic acid standard, 0.05 g of ascorbic acid (Merck) was dissolved in 0.4% oxalic acid and made up to 50 ml. The solution was titrated with 2,6-dichlorophenol indophenols for calculation of vitamin C content.
2) The chemicals for reducing and total sugar analysis
   (1) DNS, 10 g 3,5-Dinitrosalicylic acid (Sigma-Aldrich) were dissolved in 200 ml of 2 M NaOH (Labscan) with heat and stirred for completely dissolved. The 300 g sodium potassium tartrate tetrahydrate (Labscan) was dissolved in 500 ml distilled water. Then mixed the solution together and made up to 1,000 ml
   (2) 1.5 M H_2SO_4 , 81.59 ml sulfuric acid (Labscan) were dissolved in distilled water and made up to 1,000 ml
   (3) 10% NaOH, 100 g sodium hydroxide (Labscan) were dissolved in distilled water and made up to 1,000 ml
3) The chemicals for glucosinolate analysis
   (1) 5 mM sinigrin, 0.20 g sinigrin (Sigma-aldrich) was dissolved in distilled water and made up to 100 ml
   (2) 0.4 M barium acetate, 10.21 g barium acetate (QReC) were dissolved in distilled water and made up to 100 ml
   (3) Standards for HPLC (Sigma-aldrich): gluconapin, progoitrin, epiprogoitrin, glucoiberin, glucoraphanin gluconasturtiin
   (4) Diethylaminoethyl (DEAE) Sephadex A25 (Sigma-Aldrich)
4) The chemicals for crude fiber analysis
   (1) 0.128 M H_2SO_4, 6.96 ml sulfuric acid (Labscan) were dissolved in distilled water and made up to 1,000 ml

(2) 0.313 M NaOH, 12.52 g sodium hydroxide (Labscan) were dissolved in distilled water and made up to 1,000 ml

(3) Acetone (Labscan)

3.2 Methods

Experiment 1 Effects of seasons and harvesting time on senescence in pak-choi

The organic pak-choi were harvested from the planting plot at three particular times (morning, afternoon and evening) in three growing seasons (winter, summer and rainy). Then they were brought to the Royal Project packinghouse, located about 87 km from the farmer's plot in Muang district, Chiang Mai province where the vegetable was sorted for uniformity, trimmed, and packed in 25×40 cm perforated polyethylene bags with 18, 0.8 cm diameter holes. Each bag contained 300 g pak-choi samples. After that, the samples were placed at normal room conditions (25-30 ºC, 49-70% RH). Recordings were made daily on physiological changes of pak-choi until senescence's occur (yellow leaf).

Experimental design: The experimental design was 3×3 factorials in completely randomized design (CRD) with two factors and four replications. The first factor was growing season and the second was harvesting time as follows:

Growing seasons: winter (November-December), summer (February-March) and rainy season (June-July)

Harvesting times: morning (5.30-7.30), afternoon (12.00-14.00) and evening (16.30-18.30)

Statistical Analysis: A factorial in CRD was used with four replications. Statistical analysis used the general linear model and compared mean by one-way ANOVA with the SPSS program at a 95% confidence level.

Data collection:

Pre-harvest: The data recorded in farmer's plot included:

1) Temperature (ºC), relative humidity (%RH) and light intensity (lux) in the planting plot was recorded by data logger.

2) Fresh and dry weight - 12 plant samples were weighed and dried by hot air oven every week.
3) Content of reducing sugar and total sugar - 12 plant samples were analyzed for reducing and total sugar content every week.
4) Color leaf change - 12 plant samples were examined by a SPAD 502 Plus chlorophyll meter every week.

Harvesting: The data recorded during harvesting included:

1) Temperature of 12 plant samples in each treatment were measured by infrared thermometer.
2) When harvesting, ambient temperature, relative humidity and light intensity were recorded by data logger.

After harvest: The data recorded in laboratory every day included:

1) Weight loss
2) Contents of reducing sugar and total sugar (James, 1995)
3) Content of vitamin C (Ranganna, 1986)
4) Content of glucosinolate (Chen *et al*., 2008)
5) Content of crude fiber (A.O.A.C., 2005)
6) Leaf color
7) Respiration rate
8) Ethylene production rate
9) Storage life and produce appearance: yellow leaves, wilt, etc.

<u>Experiment 2</u> Effects of vacuum cooling on delay of senescence of organic pak-choi in each season

Pak-choi samples were grown under the organic standards of ACT (IFOAM Accredited). They were cultivated in a farmer's greenhouse in Muang Ang village, Chom Thong district, Chiang Mai province, Thailand. Fifteen-day old pak-choi seedlings were transplanted. Then 24-27 days after transplanting, organic pak-choi were harvested from the planting plot at 5.30-7.30 in three growing seasons (winter, summer and rainy). Then they were brought to the Royal Project packinghouse, located about 87 km from the farmer's plot in Muang district, Chiang Mai province where the vegetable

was sorted for similarity, trimmed, and packed in 25×40 cm perforated polyethylene bags with 18, 0.8 cm diameter holes. Each bag contained 300 g pak-choi samples. One part of the samples was precooled under vacuum cooling at a 6.0 millibar bleed pressure with 5 min soak time then stored at 8 ºC and 70-80% RH. Another part was stored at 8 ºC immediately. The produce from vacuum cooling was compared with the one from non-vacuum cooling.

Experimental design: An independent-sample t-test was used with four replications. The factors were vacuum cooling and non-vacuum cooling.

Statistical analysis: The average daily data between two treatments and 4 replications were compared by the independent-sample T-test with the SPSS program at a 95% confidence level.

Data collection:

1) Weight loss
2) Contents of reducing sugar and total sugar (James, 1995)
3) Content of vitamin C (Ranganna, 1986)
4) Content of glucosinolate (Chen *et al*., 2008)
5) Content of crude fiber (A.O.A.C., 2005)
6) Leaf color
7) Respiration rate
8) Ethylene production rate
9) Shelf life and produce appearance: yellow leaves, wilt, etc.

3.3 General methods

3.3.1 Weight loss

The pak-choi samples were weighed and weight loss was calculated.

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

3.3.2 Content of reducing sugar and total sugar

The contents of reducing sugar and total sugar were determined based on the technique of James (1995). The samples for sugar contents were kept by frozen before analyzing.

Content of reducing sugar, 3 g of sample were weighed and 50 ml of distilled water were added. The solution was warmed in a water bath at 55 ºC for 10 min. Then it was filtered and made up to 100 ml with distilled water. One ml of the solution was pipetted and 1 ml DNS and 2 ml distilled water were added. The solution was boiled in a water bath at 100 ºC for 5 min, then cooled and adjusted to a volume of 25 ml with distilled water. The absorbance of the sample solution was read at a wavelength of 540 nm using a spectrophotometer. Then reducing sugar content was calculated with a standard curve. The standard curve was made from solutions of glucose at the concentrations were 0, 0.1, 0.5, 1.0, and 10 mg/ml (Figure B1-B3).

Content of total sugar, 1 g of sample was weighed and 10 ml of 1.5 M H_2SO_4 were added. The sample solution was boiled in a water bath at 100 ºC for 20 min, cooled and 12 ml of 10% NaOH were added. Then it was filtered and made up to 100 ml with distilled water. One ml of the solution was pipetted and 1 ml DNS and 2 ml distilled water were added. The sample solution was boiled in a water bath at 100 ºC for 5 min then cooled and adjusted to a volume of 25 ml with distilled water. Absorbance of the solution was read at 540 nm using a spectrophotometer. Then total sugar content was calculated with a standard curve. The standard curve was made from solutions of glucose at the concentration were 0, 0.1, 0.5, 1.0, and 10 mg/ml (Figure B1-B3).

3.3.3 Content of vitamin C

Vitamin C content was analyzed by the indophenol titration method (Ranganna, 1986). About 10 g of ground pak-choi sample were weighed then 0.4% oxalic acid was added and made up to a volume of 100 ml. The sample solution was filtered with Whatman No.1 paper. Ten ml of the filtered sample solution was titrated with 0.04% 2,6-dichlorophenol indophenol until the end point. The volume of 2,6-dichlorophenol indophenol solution at the end point was used to calculate ascorbic acid content.

Volume of indophenol dye = a ml, Ascorbic acid = 1 mg

Volume of indophenol dye in sample = b ml, Ascorbic acid = (1 × b)/a = c mg

Sample solution 10 ml, Ascorbic acid = c mg

Sample solution 100 ml, Ascorbic acid = (c × 100)/10 = d mg

Pak-choi sample 10 g, Ascorbic acid = d mg

Pak-choi sample 100 g, Ascorbic acid = (d × 100)/10 = e mg/100 g FW

3.3.4 Content of glucosinolate (GS)

The content of glucosinolate was measured according to the method of Chen *et al*. (2008). A 0.25 g freeze-dried powder sample of pak-choi was preheated for 5 min at 75 ºC in a water bath. Then 4 ml of 70% boiling methanol were added, and extraction was conducted at 75 ºC in a water bath for 10 min. For internal standardization, 200 ml of 5 mM sinigirin were added to one of the duplicates before extraction. One ml of 0.4 M barium acetate was rapidly added to the preheated sample and the test tube was vortexed for several seconds. The sample was centrifuged at 4,000 rpm for 10 min at room temperature. The supernatant was collected and the pellet was re-extracted twice with 3 ml of 70% boiling methanol. The three supernatants were combined and made up to 10 ml with 70% methanol as a sample extract. Five ml of the sample extract were loaded onto activated DEAE Sephadex A25 in a vacuum processor and allowed to desulfate overnight with arylsulfatase. The resultant desulfo-glucosinolate (desulfo-GS) was eluted with 2.5 ml of ultrapure water and stored at -20 ºC prior to separation and analysis by HPLC. Then the GS content was calculated with a standard curve. The standard curve was made from solutions of sinigrin hydrate at the concentrations of 1, 10, and 100 µg/ml (Figure B4-B6).

3.3.5 Content of crude fiber

The content of crude fiber was determined according to the method of A.O.A.C. (2005). The crucible was heated at 105 ºC for 1 h and cooled in desiccator (A). Fiber bags were heated at 105 ºC for 1 h and cooled in desiccator (B). One g of dried pak-choi sample (C) was placed in a fiber bag, boiled in 0.128 M H_2SO_4 for 30 min and washed with warm water. The sample was reboiled in 0.313 M NaOH for 30 min and washed with warm water and acetone. Then the sample was dried at 105 ºC for 4 h and cooled in a

desiccator. The dry sample was weighed (D), put in a crucible, burned at 600 ºC for 4 h and cooled in a desiccator. The ash was weighed (E) to calculate crude fiber content.

$$\text{Crude fiber (\%)} = \frac{(A + D - B) - E}{C} \times 100$$

3.3.6 Leaf color was measured every day using a chlorophyll meter (SPAD-502 Plus). The measured value is SPAD unit.

3.3.7 Respiration rate

The produce sample was weighed then put in a plastic box. The plastic box was connected with a flow board (Figure 3.1) and stored with the treatments' conditions. The flow rate was measured by air flow meter (Figure 3.2) and CO_2 was detected by a gas detector (Bridge Analyzers). Both of flow rate and the amount of CO_2 were calculated for respiration rate according to the method of Smith (1995).

$$\text{Respiration rate (mg } CO_2\text{/kg-h)} = \frac{(\%CO_2 - \text{blank}\%CO_2) \times \text{Flow rate (ml/min)} \times 321750 \text{ mg/kg/h}}{\text{Weight (g)} \times (273 + \text{Measured flow rate temp } ^oC)}$$

3.3.8 Ethylene production rate

Ethylene production rate was determined by storing pak-choi samples in sealed 1,500 ml plastic boxes for 2 h. One ml of gas in the plastic box was collected and injected into a gas chromatography (GC) machine under N_2 carrier gas, with a 100 ºC column oven and 50 ºC injector. Ethylene production rate was calculated using the peak (area) reading sample data and ethylene standard, sample weight, time use and volume of the plastic box. The unit of ethylene production rate is µl/g/h.

$$\text{Ethylene production rate (µl/g-h)} = \frac{\text{free volume}^{1/} \text{ (ml)} \times \text{ethylene}^{2/} \text{ (ppm)}}{\text{Sample weight (g)} \times \text{Sealed time (h)}}$$

[1/]free volume = Volume of plastic box – Volume of sample

[2/]ethylene calculation; calculate the area under graph between standard and samples.

The area under graph of standard A unit had ethylene = B ppm

The area under graph of sample C unit had ethylene = (C×B)/A ppm

3.3.9 Storage life or shelf life and produce appearance

Storage life or shelf life was determined by visual quality such as leaves yellowing, wilting, and rotten. One trained-panel was used for assessment. When the leaves started turning yellow or withered, the shelf life had expired. The sample was reject if those symptoms occurred.

3.4 Locations

1. Farmer's greenhouse at Muang Ang village (Inthanon Royal Project Development Center), Chom Thong district, Chiang Mai province
2. The Royal Project Produce Center, Muang district, Chiang Mai province
3. Postharvest research unit, Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai province
4. The central laboratory, Faculty of Agriculture, Chiang Mai University, Chiang Mai province