CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Equipment

- Balance (KERN 510)
- Vortex mixer (IKA MS1)
- Hot plate
- Centrifuge (Hettich EBA 8S)
- Spectrophotometer (Spectronic Genesys 5)
- Portable area meter (LI 3000 A)
- Oven (Venticell 111)
- Digesting system
- Atomic Absorption Spectrophotometer (Varian Spectr AA-200, Australia)

Supplies

- Mortar and pestle
- Micropipette
- Pipette
- Beaker
- Test tube
- Forceps
- Parafilm
- Dropper
- Filter paper (Whatmann # 1)
- Flask and Funnel
- Cylinder
- Volumetric flask
- Cork boror
- Cuvette
- Petri dish (40x10 mm.)

- Transparent plastic box (19x38x10 cm.)
- Vial

Plant materials

In this study, four plant species were selected. Two of them are well-known vegetables for people in South East Asia, whereas another two species are considered as weeds.

1. White-flowering Kangkong (*Ipomoea aquatica* Forsk) or Phakbung Chin (Thai) The plant originated in tropical Asia (possibly India). It can be found in many parts of the world, but only in South and South East Asia considered Kangkong as an important leafy vegetable (Siemonsma and Kasem Pileuk, 1994).

2. Chinese Kale (*Brassica oleracea* L. cv. group Chinese Kale) or Phakkhana (Thai) Chinese Kale is a cultigen native to southern and central China. It is now widely cultivated and popular in South East Asia (Siemonsma and Kasem Pileuk, 1994).

Seeds of these vegetables were purchased from Department of Horticulture, Faculty of Agriculture, Kasetsart University.

3. False daisy (*Eclipta prostrata* (L.) Hassk.) or Kameng (Thai) It is an annual herb found in paddy and upland crop area and along canals as well as ditches. It prefers both wet and dry conditions. This weed distributes throughout Thailand (Noda et al., 1986). Seeds of this plant were collected from the same population at Soi Ladprao 101, Bangkapi district, Bangkok.

4. Swollen finger grass (*Chloris barbata* Sw.) or Yaa Rangnok (Thai) This annual grass, propagated by seeds, has rather short life span. It prefers dry conditions, often in waste lands, roadside and pasture. It can be found throughout the country (Noda et al., 1986). Seeds of this plant were collected from the same population in Chulalongkorn University campus.

Culture conditions

Seeds of plant materials were germinated on a moistened sand basket. Seedlings of vegetables were transferred to plastic pots containing 1 litre of an aerated half concentration of Hoagland's solution within 7 days after sowing, whereas seedlings of weeds were grown in the sand basket not less than 14 days before transplanting to the nutrient solution due to weakness of

young seedlings and difficulty when handling. The solution was prepared by tap water and then the pH of the solution was adjusted to 5.7 with 0.1N HCl.

Plants were placed in phytotron (30/27 ^{0}C day/night ; 70/90 % RH day/night ; 60-80 μmol $m^{-2}S^{-1}$, 16 h day^{-1})

The solution was replenished with tap water and adjusted to pH 5.5-6.0 twice a week. About 1 month after transplanting, the solution was renewed by half concentration of Hoagland's solution using distilled water to prevent the precipitation of metal ion. The solution pH was also adjusted to 5.7 by 0.1N NaOH. Cd was added as $CdCl_2$ to produce final concentration of 5 and 20 ppm. Treatments with 5 ppm Cd were analysed after 2, 11 and 20 days of exposure to observed the long term effects of Cd. Even so, to observe the severe effects of Cd at high concentration, the 20 ppm treatments were analysed after 2, 5 and 8 days of exposure because *I. aquatica*, the most susceptible species, could no longer stand the toxic effects of heavy metal. In addition, at the 8th and the 20th day of exposure for 20 and 5 ppm Cd treatment respectively, some plants were transferred to normal nutrient solution. The experiments were also performed at the 7th and the 14th day after transfer. The nutrient solution containing Cd was renewed twice a week. However, the one without Cd was only replenished by solution of one-tenth concentration, and pH of the solution was adjusted to between 5.5-6.0. Three replications were prepared, giving a total of 30 pots for each treatment.

3.2 Methods

This study can be divided into 3 parts as follows.

3.2.1 Study on the physiology of plants during Cd stress and after stress was released.

1) Proline, chlorophyll and Relative Water Content (RWC) were determined.

2) Study on the growth of plants focused on the number of leaves, leaf area and Root to Shoot Ratio.

3.2.2 Study on cadmium accumulation. Plant materials from the second day of exposure, the last day of exposure and the last day of experiment were collected.

3.2.3 Statistical analysis

3.2.1 Study on the physiology of plants

The same leaves were used for analysis. However, the number of leaves used for this study differed from plant to plant.

I. aquatica : The first unfolded leaf to the fifth leaf from the tip were used.

B. oleracea : The first unfolded leaf to the third leaf from the tip were used.

E. prostrata : The first unfolded pair of leaves to the third pair of leaves from the main shoot were used.

C. barbata : The first fully expanded leaf to the third leaf from the main shoot were used.

Proline Determination

The method for determination of free proline was modified from the acid-ninhydrin method developed by Bates et al. (1973). 0.1 g of leaves was ground by mortar and pestle with acid-washed sand, and 12.5 cm^3 of 3% aqueous sulfosalicylic acid was gradually added. The homogenized sample was filtered through Whatmann No. 1 filter paper. Two cm^3 of filtrate was let to react with 0.15 g amberlite resin, while agitating the test tube using vortex mixer, in order to prevent the interference of other amino acids such as ornithine, lysine and hydroxylysine. Two cm^3 of glacial acetic acid and 2 cm^3 of acid ninhydrin (1.25 g ninhydrin dissolved in 30 cm^3 glacial acetic acid and 30 cm^3 6 M H_3PO_4) were added to the filtrate. The mixture was heated in boiling water for 1 hour. The pale yellow mixture would turn red when proline reacted with inhydrin at approximately pH 1.0 during boiling. However, the red mixture would not be noticed if the sample contained few proline. Then, the reaction was immediately stopped by dipping the tube into an ice bath.

After 5 to 10 minutes of cooling, 4 cm^3 of the reaction product was extracted by thorough mixing of an equal amount of toluene. The impermissible solution was left 1 to 2 minutes. Then, 2.5 cm^3 of supernatant was filled in a tube and was centrifuged with 5,000 rpm for 10 minutes. The 2 cm^3 upper solution was used to determine the absorbance at 520 nm by spectrophotometer using toluene as a blank. The proline content was calculated from the standard curve and the following equation.

$$\mu mol\ proline\ g^{-1}\ fw = \frac{(\mu g\ proline\ cm^{-3} \times cm^{3}\ toluene)}{115.5\ \mu g\ \mu mol^{-1}} \times \frac{2}{(g\ FW\ sample)}$$

Chlorophyll determination

The method for determination of chlorophyll was modified from the method developed by Arnon (1949). 0.05 gm of leaves was soaked in 5 cm^3 of 80% acetone in the sealed test tube. The tube was kept in the dark for 24 hours at room temperature. The absorbance of the solution was measured by spectrophotometer at 645 and 663 nm. Chl a, chl b, and chl (a+b) concentration were calculated from the formula compiled by Arnon (1949) and modified by Bruinsma (1961).

$$Chl\ a = 12.72\ A_{663} - 2.58\ A_{645}$$

$$Chl\ b = 22.87\ A_{645} - 4.67\ A_{663}$$

$$Chl\ (a+b) = 8.05\ A_{663} + 20.29\ A_{645}$$

Relative water content **(RWC)**

The fresh weight (FW) of five leaf discs of the dicots and seven pieces of leaves of *C. barbata*, the monocot, cut by a 0.83 cm. diameter cork borer were measured immediately after cutting. Then, the samples were floated on 3 cm^3 distilled water in covered 40x10 mm. petri dish which was placed in closed transparent plastic box containing water to assure the high humidity during 7 hours of soaking at room temperature and under fluorescent light. To determine the turgid weight (TW), the surface dried samples were put in a vial and their weight were measured. The vial was placed in 80^0C oven for 2 days, and then dry weight (DW) was determined. RWC was calculated from the formula (Meesilpa, 1995).

$$RWC\ (\%) = \frac{FW-DW}{TW-DW} \times 100$$

Study on the growth of plants

<u>The number of leaves</u>

The dead leaves and the young folded leaves of dicots and the immature leaves of the monocot were ignored when counting the number of leaves.

Leaf area

The area of all the leaves apart from the ones as mentioned above was measured by portable area meter. The measurement was repeated three times, but only one plant per pot was determined.

Root to Shoot Ratio

In this study the plant part above the position of cotyledons was considered as shoot for dicots, whereas the base including culm and the leaves of the monocot were regarded as shoot. The plant cells were killed in 105^0C oven for 30 minutes and this sample was dried at 80^0C for the next 44 hours. The dry weight of each plant part was measured and used for calculation of root to shoot ratio.

3.2.2 Study on cadmium accumulation

Dry samples were separated into roots, stems and leaves except *C. barbata* of which culms were included in the leaf part. 0.2 gm of ground sample was digested with mixed acid (5 cm^3 of HNO_3, 1 cm^3 of $HClO_4$ and 0.5 cm^3 of H_2SO_4). Heating was prolonged until approximately 15 minutes after the first appearance of white fume. The solution was adjusted to 25 cm^3 and kept in plastic bottle (Allen, 1989). The Cd content was analysed by Atomic Absorption Spectrophotometer.

3.2.3 Statistical Analysis

The design of the pot experiment was a two-factorial replicated in CRD. Data was subjected to ANOVA with Cd treatment and plant species as the two experiment factors. Mean comparisons were made using Duncan's Multiple Range Test (DMRT). Correlations among treatments and parameters were also calculated.