CHAPTER III

METHODOLOGY

In order to achieve the objectives of this work for investigation the best practice for rice planting to reduce cadmium uptake, eight comparisons of treatments were carried in a greenhouse pot experiment as following details in Table 3.1

Table 3.1: Experimental Pot Treatments

Treatment	Description	Treatment Code
Current Farmer Practice (Control)	Drainage twice during the rice growth period, namely at mid-panicle development (1 week) and at heading	T1
Flooded	Maintenance of standing water in pots to a depth of 10cm until the hard-dough stage	T2
Farmer Practice + Rice Straw	Incorporation of rice straw and drainage twice during the rice growth period, namely at mid-panicle development (1 week) and at heading	T3
Flooded + Rice Straw	Incorporation of rice straw and maintenance of standing water in pots to a depth of 10cm until the hard-dough stage	T4
Farmer Practice + Liming	Liming to pH 7.5 and drainage twice during the rice growth period, namely at mid-panicle development (1 week) and at heading	T5
Flooded + Liming	Liming to pH 7.5 and maintenance of standing water in pots to a depth of 10cm until the hard-dough stage	T6
Farmer Practice + Liming + Rice Straw	Incorporation of rice straw, liming to pH 7.5 and drainage twice during the rice growth period, namely at mid-panicle development (1 week) and at heading	T7
Flooded + Liming + Rice Straw	Incorporation of rice straw, liming to pH 7.5 and maintenance of standing water in pots to a depth of 10cm until the hard-dough stage	T8

Each treatment consisted of 3 replicates, so the pot experiments totaled together 24 pots for 8 treatments.

3.1 Rice pots setup and preparation

3.1.1 Soil preparation for pot experiment

In the study area, there are many levels of cadmium contamination. The highest frequency distribution is the soils which have cadmium concentration vary from 0 to 10 mg/kg and pH vary from 6.0 to 6.5 units. Soil collection and preparation were carried out as following steps;

1) Four sample points were taken in each of 4 fields of selected zone, which have cadmium concentration of 0 to 10 mg/kg (16 random sample points)
2) 25-30 kg of soil samples (field weight) was taken at 20 cm depth
3) Soil samples were mixed, dried and put through a soil crusher
4) Crushed soil samples were thoroughly mixed to achieve homogeneity
5) Prepared study soils had initial cadmium concentration of 5 mg/kg

3.1.2 Rice pots preparation

Prepared study soils containing initial cadmium concentrations of 5 mg/kg with pH from 6 to 6.5 were mixed with necessary materials selected for each treatment. The rice straw for treatment T3, T4, T7, and T8 was added at a ratio of 255 grams per pot. The liming agent for treatment T5, T6, T7, and T8, for the adjustment of the pH to 7.5 was calcium oxide, which would readily be available to Mae Sot farmers. Calcium oxide (CaO) was added at the ratio of 0.0203 grams per pot approximately. The fertilizers used in this research, contained 3 main fertilizer types; ammonium sulfate ($(NH_4)_2SO_4$), chemical formula 21-0-0; triple super phosphate (TSP), chemical formula 0-46-0; and potassium chloride (KCl), chemical formula 0-0-60; which were used as the sources of nitrogen, phosphorus, and potassium, respectively. The rates of fertilizers added in each pot are 0.4464 grams per pot for ammonium sulfate, 0.6114 grams per pot for triple super phosphate, and 0.3125 grams per pot for potassium chloride. The prepared study soils were transferred in to experimental appropriate labeled pots as the final steps.

Rice pots preparation was operated as described in the following steps:

1. Pre-soaked rice seeds
2. Sow rice seed in nursery pots
3. Separated the selected contaminated soils into 8 groups for 8 treatments which described above.
4. Mixed the fertilizer to all separated groups soils at the rate of 0.4464 grams per pot for ammonium sulfate, 0.6114 grams per pot for triple super phosphate, and 0.3125 grams per pot for potassium chloride
5. Mixed the liming agent (calcium oxide) at the ratio of 0.0203 grams per pot to treatments T5, T6, T7, and T8 for adjust the pH to 7.5
6. Mixed the rice straw to treatments T3, T4, T7, and T8 at a rate of 255 grams per pot
7. Transferred soil from each treatment replicates to experimental appropriately labeled pots
8. Wet up all soils (using tap water from the greenhouse) sufficient to allow approximately 5 cm of water surface above the soil surface and continue everyday to maintain the water level in every pot.
9. Transplanted 3 rice plants from nursery pots to each pot at 14 days after sowing
10. Removed the weakest rice plant from each pot at 7 days after transplanting (2 rice plants remaining in each pot)

Rice pots in the greenhouse at the Department of Agriculture, Kasetsart University is showed in Figure 3.1. Rice seed used in the experiment was Khao Dowk Mali 105 (KDML 105). Rice seed samples were thoroughly mixed to obtain the complete mix stage before being used in the experiment. Several analyze was conducted and showed that rice seed samples containing cadmium concentration of 1 mg/kg.

Figure 3.1 **Rice pots in the greenhouse at the Department of Agriculture, Kasetsart University**

3.2 Samples collection

After transplanting rice plants, the study consisted of 2 periods of sampling, during grain fill stage (drainage period, 75 days after transplanting) and during harvest period (125 days after transplanting) for all type of samples.

3.2.1 Soil solution collection

There were 2 periods of samples collection, which are the first and the second drainage periods. First sampling period, soil solution was collected 3 days before drainage (75 days after transplanting) and sampling was continuing everyday until redox potential was stable after reflooding (only treatment T1, T3, T5, and T7 were drained at first drainage period). Second sampling period, soil solution was collected 3 days before second drainage (125 days after trandplanting) and sampling will continue everyday to 10 days after drainage.

Soil solution sampling method

A rhizon sampler will be placed permanently in the middle of the rhizosphere between 2 rice plant roots colonies. The rhizon sampler is long enough to be located about 20 cm above the water surface. At the end of each rhizon sampler are screws, so a needle can connect them to a vacuum tube for sampling soil solution. The needles was used only once to connect rhizon samplers to vacuum tubes at the end of rhizon sampler. When a vacuum tube was connected to rhizon sampler via needles, soil solution was sucked by vacuum tubes negative pressure. Soil solution samples were stayed in vacuum tubes until analyzed. Soil solution collection within the greenhouse is showed in figure 3.2. The rhizon sampler and vacuum tube, used to collect soil solution from rice pots are showed in figure 3.3, 3.4, and 3.5.

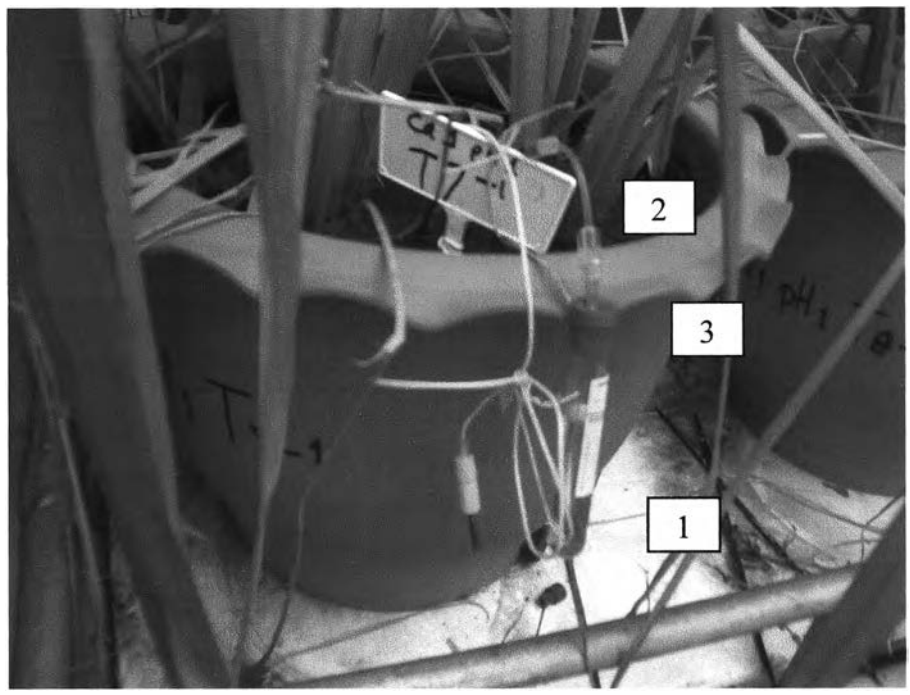

Figure 3.2 **Soil solution collections within the greenhouse, vacuum tube (1) connected to rhizon sampler (2) using needle (3).**

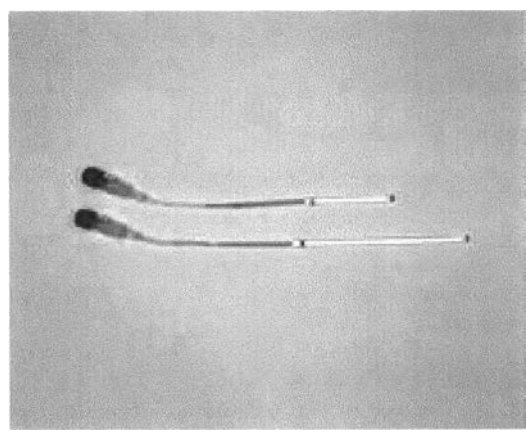

Figure 3.3 **The rhizon sampler**

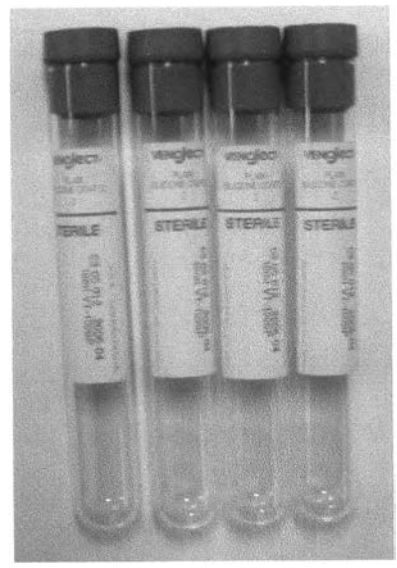

Figure 3.4: **Vacuum tube**

Figure 3.5: **The rhizon sampler connected to vacuum tube by needle**

3.2.2 Soil samples collection

Soil samples collection was also conducted for two periods, during the grain fill stage (at drainage day, 75 days after transplanting) and during harvest period (at the harvest day, 125 days after transplanting). Soil sampling during the grain fill stage was undertaken by LDD.

Soil sampling method

For each pot, one soil sample/core (0-15 cm depth and 3 cm internal diameter) were collected per pot and placed in a plastic sampling bag. All soil samples were kept in the refrigerator to maintain soil moisture until analyzing.

3.2.3 Plant samples collection

Plant samples were collected at the same day as soil samples collection. Plant samples were collected during the grain fill stage (at drainage day, 75 days after transplanting) and during harvest period (at the harvest day, 125 days after transplanting).

Plants sampling method

At both period of sampling, the first and second drainage, rice plants were collected by LDD staffs by cutting one plants of 2 plants out for analysis. So, there were one rice plant left after first drainage and that plant was cut for analyzing after harvest. Plant samples consist of 2 parts of rice plants, which are stems and leaves. Each part was analyzed for heavy metals concentrations individually.

3.3 Samples preparation

3.3.1 Soil solution samples preparation

Soil solution samples were preserved by conc. Nitric acid at 5% by volume as fast as possible after sampling to fix metals in the vacuum tube.

3.3.2 Soil samples preparation

Previous researches have shown that total digestion of soil samples to measure total cadmium in soil samples is a non-significant relation to cadmium uptake by rice plants. This is because there are many factors which affect cadmium uptake by rice plants; such as, zinc concentration, and iron concentration in soils, soil pH, and soil redox potential. Furthermore, many forms of cadmium in soils, such as precipitation form or complex form cannot be absorbed, nor have very low phyto-availability to rice plant. The calcium chloride extraction method is believed to be appropriate for prediction the cadmium concentration in absorbable form. In addition, calcium chloride extraction technique was selected for this research to analyze cadmium, zinc, manganese, and iron concentrations in soil samples.

Soil samples preparation by calcium chloride extraction method

1. Remove rice roots, rice straw and others impurity particles from soil samples
2. Soil samples had been weight 10g without drying (moist soil samples) and brought it into 60 ml bottle.
3. Calcium chloride extracting solution were prepared by weight 7.351g of calcium chloride and then made up volume to 1000 ml
4. 50 ml of calcium chloride extracting solution was added into 60 ml bottle and shook for 4 hr
5 After finish shaking, samples were immediately removed and then filtered samples with Whatman no.42 filter paper to appropriate labeled bottle.

The standard solutions were prepared using 0.05 M calcium chloride extracting solution as a solvent.

3.3.3 Plant samples preparation

The digestion of plant samples before measurement for heavy metals was carried out by using microwave assisted nitric: perchloric acid (3: 1) digestion method (Johnson et al., 1959). The details are as follow.

1. 0.5 g of dried and grounded plant samples were weighted and transferred into microwave vessel.
2. 10 ml of nitric 3: 1 perchloric acid was added into microwave vessels.
3. All samples were heated with microwave at 200°c for 20 minutes then bring samples out from microwave.
4. All samples were kept in room temperature to cool down and then added 1 ml of hydrochloric acid.
5. All samples were reheated with microwave at 200 °C for another 10 minutes.
6. Samples were brought out and cool down then filtered with Whatman no.42 filter paper into appropriate labeled bottle.

3.4 Sample analysis

Heavy metal concentrations (Cd, Fe, Mn, and Zn) in, prepared soil samples, were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) While the prepared soil solution samples and plant samples were analyzed be Flame Atomic Absorption Spectrometry (FAAS), and Graphite Furnace Atomic Absorption Spectrometry (GFAAS) depending on their concentration since the detection limit of GFAAS is much lower as compared to ICP and FAAS. Soil samples were analyzed for 3 replicates and plant samples were analyzed for 2 replicates.

3.5 Redox and pH measuring

3.5.1 Redox potential measurement in soil

Redox potential of each pot was analyzed using the redox meter. One of the ends of the reference electrode was permanently placed at the rhizosphere zone between 2 rice plant roots colonies in every pot. The redox probe was placed in each pot at about 1.5-2 cm depth from soil surface and then the other end of the electrode will be connected to the redox meter. The value can be read from the redox meter within the greenhouse. Redox meter, used to measure redox potential in soil within the greenhouse is showed in figure 3.6.

Figure 3.6: **Redox meter, used for redox potential measuring**

3.5.2 Measurement of pH

pH values of soil and soil solution were measured in the rhizosphere zone. At least 2 ml of soil solution from the rhizosphere was sucked by the vacuum tube after soil solution sampling and immediately measured with the pH meter within the greenhouse. pH meter used to measure pH value within the greenhouse is showed in Figure 3.7

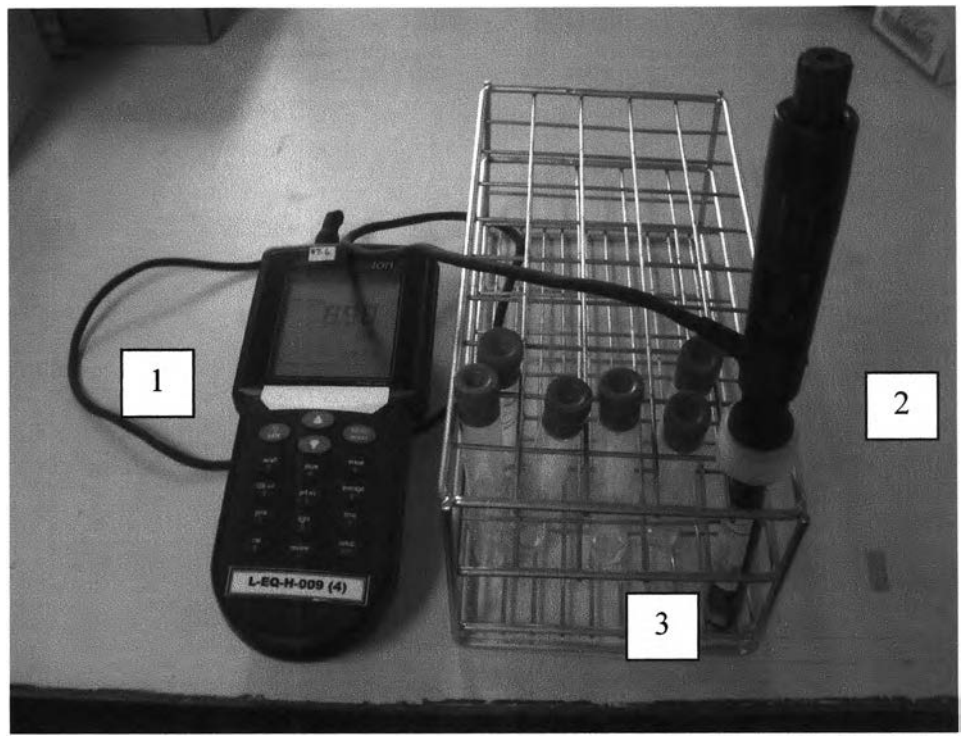

Figure 3.7: **Soil solution pH measuring within the greenhouse, pH meter (1) connected to pH probe (2) measured pH of soil solution in the vacuum tube (3).**

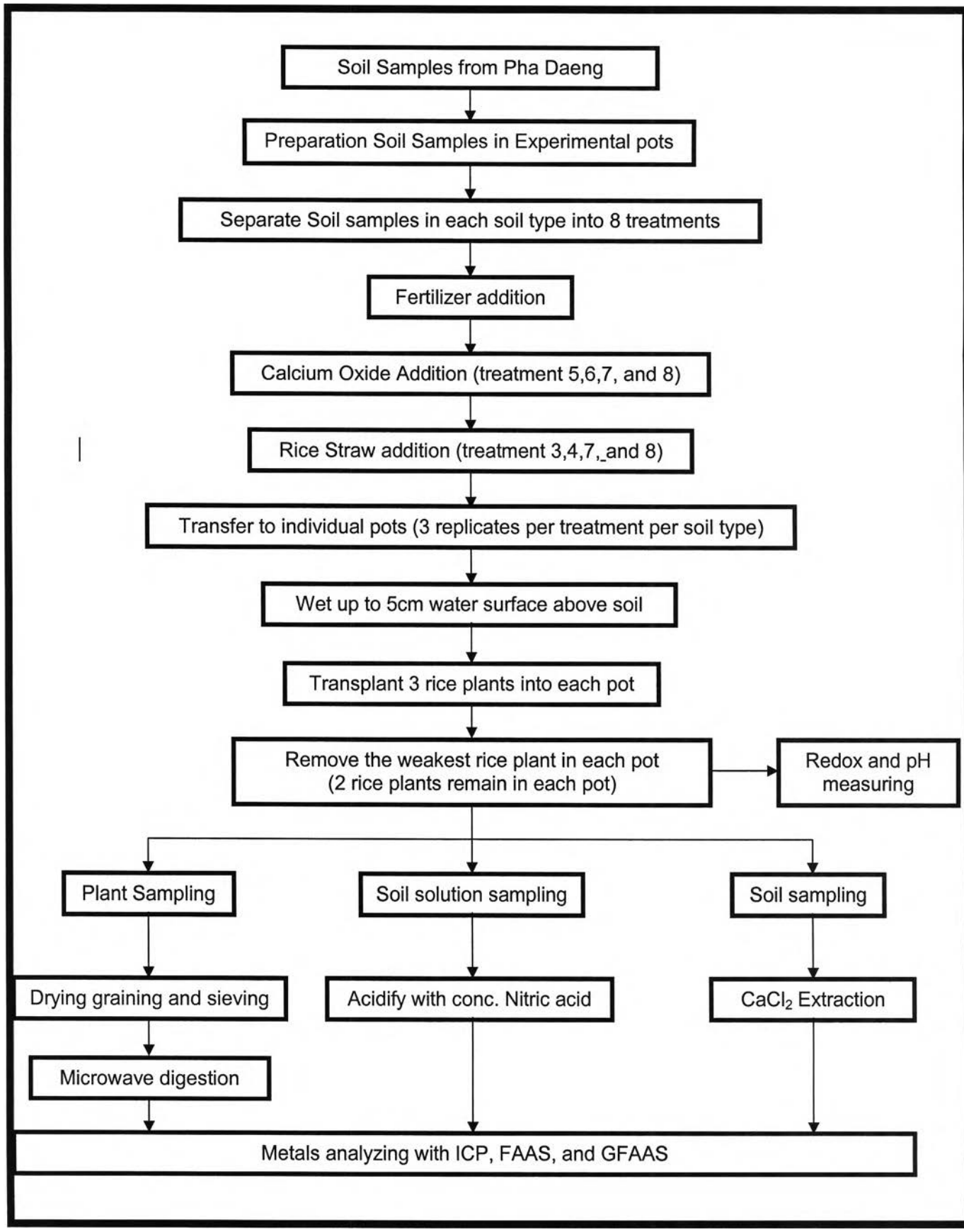

Figure 3.8: **Diagram of the step of experimental procedure of the study**