Chapter 3. Materials and Methods

3. 1. Apparatus

i. Multisolvent Delivery System, Waters 600E, Waters Chromatography Div., Milford, USA.

ii. Autosampler, Waters 717, Waters Chromatography Div., Milford, USA.

iii. Tunable UV-Visible Absorbance Detector, Waters 486, Waters Chromatography Div., Milford, USA.

iv. Chromatography Data Workstation Maxima 820, NEC PowerMate SX/16 and NEC pinwriter P6200, NEC Technologies Inc., Boxborough, USA.

v. Guard column, Guard PAK guard column holder (Part No. 88141, Waters Chromatography Div., Milford, USA), fitted with Guard PAK C_{18} μ Bondapak pricolumn inserts (Part No. 88070, Waters Chromatography Div., Milford, USA)

vi. Analytical column, spher180 RB ODS. 2; 5 μ; 250 mm × 4.6 mm. B Fitting.

vii. Ultrasonic cleaner, Branson B-2200, Connecticut, USA.

viii. Air cylinder with a pressure regulator.

ix. Helium cylinder with a pressure regulator.

x. Filter apparatus, Waters Associates, equipped with 1 liter of ground joint flask, 300 ml funnel and tabulated base.

xi. Air motor compressor vacuum pump, Waters Associates.

xii. Filter unit, Nalgene, USA, 0.20 μ.

xiii. Analytical Balance, Sartorius.

xiv. Holen Lamiair. TL2448. Denmark.

xv. Spectrophotometer, UV - 120 - 01. Shimadzu Corporation, Japan.

xvi. Rotary shaker.

xvii. Incubator. Sunnivic Controls Ltd, Great Britain.

xviii. Centrifuge. Kubota 7800. Kubota Coorparation, Japan.

xix. Automatic High-pressure Steam Sterilizer. Tomy SS-305. Tomy, Seiko Co. Ltd, Japan.

xx. Hot air sterilizer.

xxi. pH meter.

xxii. Oven.

xxiii. Refrigerator.

xxiv. Borer.

xxv. Soil auger.

3. 2. Chemicals

i. Carbaryl standard (1-naphthyl methylcarbamate) , 99.9%. Merck, Germany.

ii. Paraquat dichloride (1, 1'-Dimethyl-4,4'-bipyridinium), Sigma Chemical Co., USA.

iii. Acetonitrile, HPLC grade, 100.0 % by GC. J. T. Baker Inc, USA.

iv. Ammonia acetate, 98%. E.Merck, Darmstadt F.R. Germany.

v. Beef extract powder. Becton Dickson Microbiology Systems, USA.

vi. Yeast extract powder. Gibco Laboratories, USA.

vii. Gelysate ™ Peptone, Becton Dickinson Microbiology Systems, USA.

viii. Agar.

ix. Potassium dihydrogen phosphate, min. 97%. May & Baker Ltd., England.

x. Di-sodium hydrogen orthophosphate , 99.5 %. BDH Chemicals Ltd., England.

xi. Sodium chloride, min. 99.5 %. Merck, Germany.

xii. Magnesium chloride.

xiii. Calcium chloride.

xiv. Maganese sulfate monohydrate, min.99%. Merck, Germany.

xv. Ferrous sulfate, min. 99%. Hopkin & Williams Ltd., England.

xvi. Di-potassium hydrogen orthophosphate, 98.0 - 101.0 %. Merck, Germany.

xvii. Potassium nitrate.

xviii. Glucose.

xix. Sucrose. Difco Laboratories, USA.

xx. Soluble starch. Merck, Germany.

xxi. Deionized water, Chemistry Department, Chiang Mai University.

xxii. Distilled water, Biology Department, Chiang Mai University.

3. 3. Methods

3. 3. 1. Soil sampling

The whole sampling area is divided into 86 sub-plots (Fig 3.1), and the vegetation is mainly soybean and vegetable. Since the type of vegetation might have the effect on microbes in the soil, so samples were taken separately from the soybean field and vegetable field. Since the field had been tillaged and the soil was homogenized, so the samples were taken randomly. Five sub-plots were randomly selected from each field, and three points were randomly selected from each sub-plot. Two samples, upper layer and lower layer, were taken form each point.

The samples were taken with two steel borers shown in Fig 3.2. After the upper 10 cm layer sample was taken, the lower 10 cm layer sample was taken by the second borer. Then 15 samples from each layer were pooled together, mixed well, and the debris, roots and stones were removed. All the samples were transported to the laboratory as soon as possible.

The soil samples were divided into two parts. One part was air dried, crushed with mortar and pestle, then passed through 2 mm sieve and stored at room temperature for physical and chemical parameter analyses. The other part was stored at 4°C in the refrigerator when the microbiological analysis was not carried out.

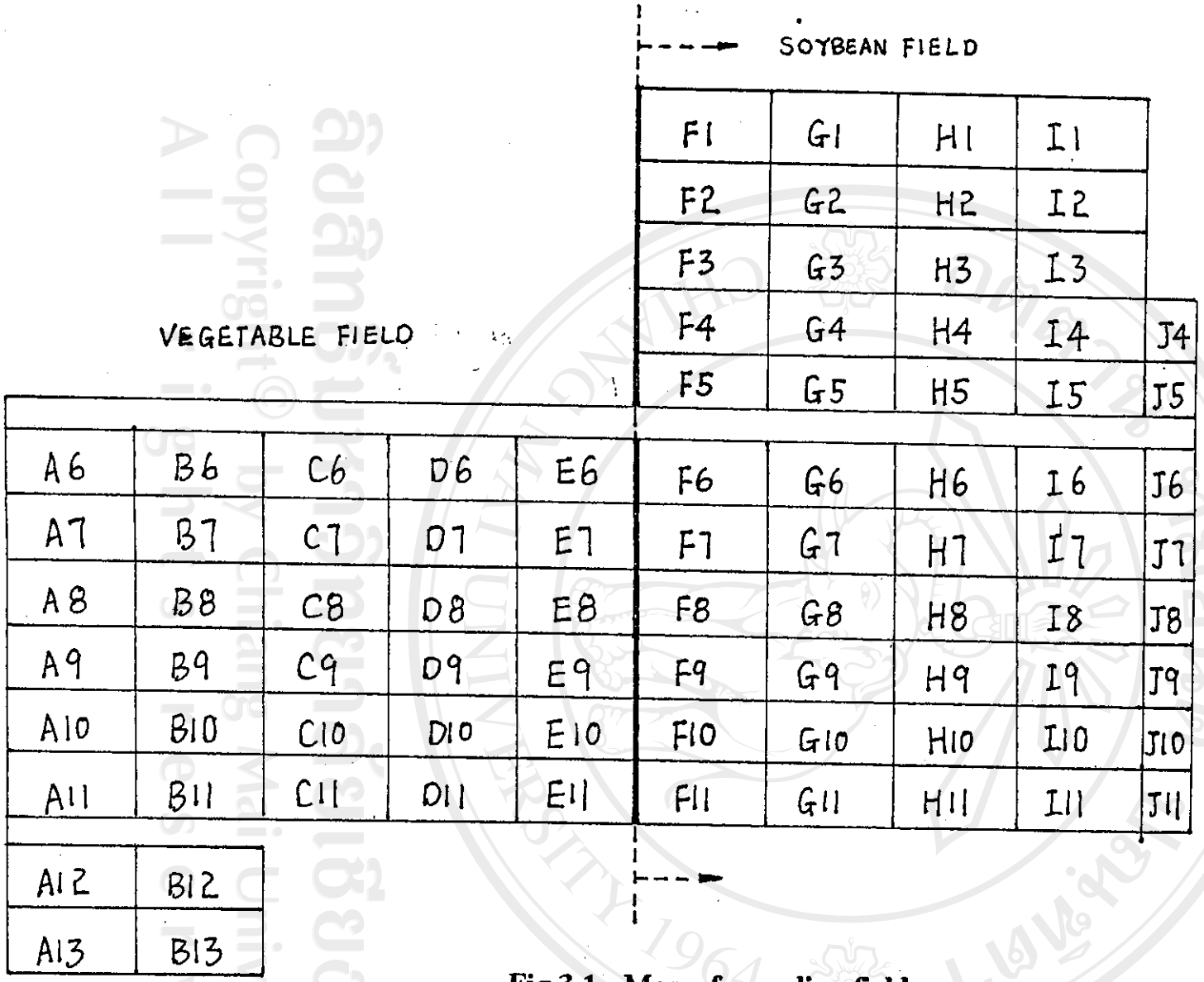

Fig 3.1. Map of sampling field.

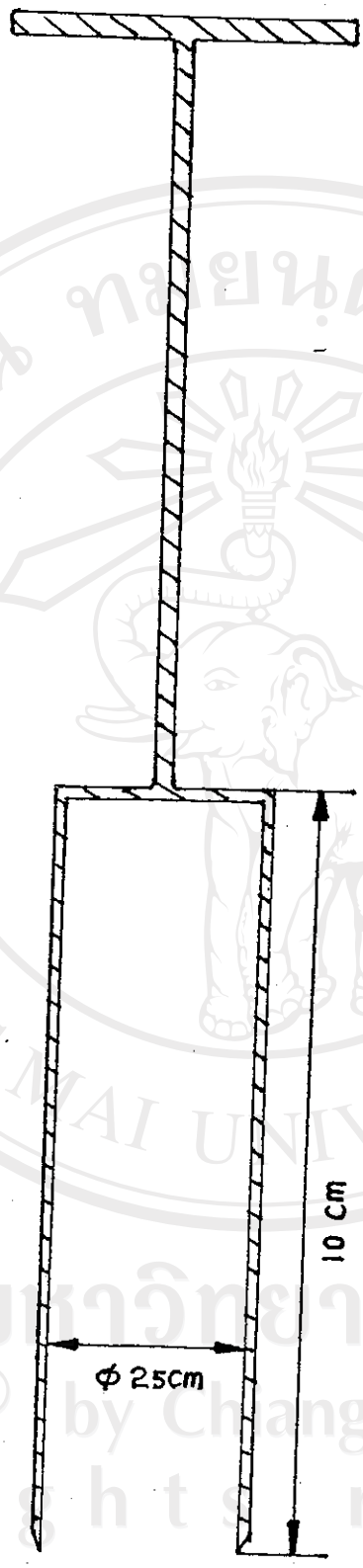

Fig 3.2. Sampling device.

3. 3. 2. Soil parameter analysis (Pramer & Schmidt, 1967)

I. Soil pH

20 g of fresh soil was weighed and put in a 125 ml conical flask, containing 40 ml of distilled water. The flask was shaken for 20 minutes. The pH was then measured.

II. Soil moisture content

About 50 g of fresh soil was added in a paper bag previously weighed, and the total weight was determined. All samples were dried in an oven at 110 °C for 24 hours, then cooled, and stored in a desiccator until weighed.

The soil moisture was calculated using the following formula:

$H_2O\ \% = [\ (W_F - W_D)/(W_D - W_B)\ ] \times 100\ \%$

W_F : total weight of the bag with fresh soil;

W_D : total weight of the bag with dry soil;

W_B : weight of the bag.

III. Water holding capacity (WHC)

WHC is measured using cup with screen bottom.

A circle of moist filter paper was placed at the bottom of the cup and the whole unit was weighed. The cup was then filled with the soil previously dried at 110°C for 24 hr. The soil was compacted by dropping the cup 10 times through a distance of approximately 3 cm, the soil surface was leveled with a spatula and the whole unit was weighed.

The cup of soil was placed in a pan containing water deep enough to wet the bottom only of the soil column. When the soil was saturated, the whole unit was placed in a humid enclosure until drainage was completed and the unit was weighed with the soil in a saturated condition.

The water holding capacity was calculated using the following formula:

$$WHC\ \% = [\ (W_S - W_D)/(W_D - W_C)\ ] \times 100\%$$

W_S : total weight of the cup with saturated soil;

W_D : total weight of the cup with dry soil;

W_C : weight of the cup with filter paper.

IV. Estimation of bacterial population

The estimation was done by dilution plate method, with soil extract agar (See Appendix B).

10 g of fresh soil was added into 250 ml conical flask containing 95 ml sterile water. The flask was shaken on a rotary shaker at 200 rpm for 10 minutes. 10 ml soil solution was taken after the flask was vigorously shaken and put in 250 ml conical flask containing 90 ml sterile water to establish a 10^{-2} dilution. This solution was serially diluted up to 10^{-5}. Then the pour plate was done with 10^{-3}, 10^{-4}, and 10^{-5} dilutions. The soil extract agar was melted and cooled down in water bath to about 50 °C. 1 ml of each dilution was put into sterile plate, and about 12 ml melted agar was poured in and mixed with the soil solution. 5 replicates were done for each dilution. After the agar was solidified, the plates were incubated at room temperature, (25°C ± 2 °C) for 7 days. The colonies appeared were counted by colony counter. The result was expressed as cfu per gram of dry soil.

The total nitrogen, exchangeable phosphorus and potassium, and soil texture were analyzed by the Soil Analysis Center, Soil Science and Conservation Department, Faculty of Agriculture, Chiang Mai University.

3. 3. 3. Enrichment, isolation and purification

The enrichment of the carbaryl - degrading microorganism was done in minimum mineral medium (Appendix B) containing carbaryl as sole carbon source. The carbaryl was dissolved in acetonitrile and added in the medium to give a concentration of 20 mg/l approximately.

0.5 g fresh soil sample previously passed through 1mm sieve was added into a 250 ml conical flask containing 100 ml enrichment medium. The flask was shaken on a rotary shaker at 200 rpm, and at room temperature. After 3 days, the culture medium was subcultured by transfering 1 ml old medium into 100 ml fresh medium, incubated at the same condition, and a sample was withdrawn from the old medium for analyzing the concentration of carbaryl by HPLC (see 3.3.8).

The isolation and purification were done with nutrient agar (Appendix B) supplemented with carbaryl at the same concentration as in the enrichment medium. The enriched culture was serially diluted and spread on the agar plates and incubated at room temperature for 3 days. The single colony appeared on the agar surface was picked and subcultured on the fresh nutrient agar plate by streak plate technique. The isolates were maintained on nutrient agar slants for further test.

3. 3. 4. Carbaryl degrading ability test

The isolate was washed from the agar slant with sterile distilled water, and 1 ml of the solution was inoculated into the enrichment medium. After 4 day incubation at room temperature on the rotary shaker at 200 rpm, the remaining carbaryl was analyzed by HPLC (see 3.3.8).

3. 3. 5. Degradation kinetics

The isolated microorganisms were first inoculated on agar slants and incubated for 3 days at room temperature. Then the agar slants were washed with sterile distilled water. The suspensions were mixed in a sterile flask with gentle shaking. 1 ml of the suspension was inoculated into 100 ml minimum mineral medium containing about 10 mg/l carbaryl in 250 ml flask, and incubated at room temperature on a rotary shaker at 200 rpm. The control flasks were done with: 1. the same medium without inoculation and 2. the minimum mineral medium with acetonitrile and inoculum except carbaryl. Samples were withdrawn at 0, 3, 6, 9, 12, 18, 24, 30, 48, and 72 hours. The growth of microorganisms was monitored in term of the turbidity of the medium by measuring the absorbance at 600nm using spectrophotometer. The concentration of carbaryl in the medium was monitored by HPLC (see 3.3.8).

3. 3. 6. Microbial degradation of carbaryl in the presence of various carbon source

1 % of the following carbon source : glucose, sucrose, and starch were added in the minimum mineral medium with 10 mg/l carbaryl, and the kinetics of degradation was analyzed by the same method in 3. 3. 5.

3. 3. 7. Microbial degradation of carbaryl in the presence of herbicide paraquat

Paraquat standard was dissolved in the minimum mineral medium with 10 mg/l carbaryl to give the concentration of 1mg/l, 10 mg/l, and 100 mg/l. Carbaryl- degrading microbes were inoculated in the 250 ml flasks containing 100 ml of the same medium. The flasks were incubated at room temperature for 3 days. The control flasks were done using the same medium with carbaryl but no paraquat. After 3 days, the concentration of carbaryl remaining in the medium were analyzed by HPLC .

3. 3. 8. Chemical analysis

The concentration of carbaryl in the medium was analyzed by HPLC (Sangphagdee, 1994) with a spher 180 RB ODS column and UV detector. The samples were centrifuged at 10,000 rpm , 4 ° C before the injection, and diluted with deionized water if necessary. The working curve ranged from 0.1000 mg/l to 2.000 mg/l and 0.0200 mg/l to 0.1000 mg/l. The operating condition was:

Mobile phase - 50 / 50 (V / V) acetonitrile and 1 mM ammonium acetate. (Appendix B) .

Flow rate - 1 ml / min.

Inject volume - 20 µl.

The confirmation test was done by injecting one standard (1 mg / 1) and one sample at wavelength 270 nm. The peak height and area ratio was calculated and compared.