CHAPTER III

MATERIALS AND METHODS

3.1 Field observation and farmer interview

Twenty soybean fields in the rubber plantations in Chamkaar Andong village, Chamkaar Andong Commune, Chamkaar Leu district, Kampong Cham province about 124 km from Phnom Penh, Eastern of Cambodia were selected (Fig. 2) as the studied sites for collection of data on farmer practices for soybean cultivation, land use history, soybean varieties used by farmers, and soybean yield by field observation and farmer interview.

3.2 Collection of soil samples and native root nodule bacteria from Cambodia

One composite soil sample was collected from each of 5 selected soybean fields having different periods of soybean cultivation. Only the top soil (0-15 cm) samples were used. The surface soil from each site was collected during March, 2008. Each soil sample was air dried before analysis of pH, organic matter, available P and exchangeable K by soil testing kit. Five soybean plants randomly selected from each site were used for nodule collection. The variety of soybean grown in each selected field was recorded. The collected root nodules from each site were kept in closed test tube containing silica gel as moisture absorbent material before isolation of root nodule bacteria in the nodules. Isolation of native root nodule bacteria in the collected nodules were done by the common method using yeast mannitol congo red agar (YMA) (Somasegaran and Hoben, 1994). Rate of colony formation in YMA,

colony morphology and colony characteristic on YMA with congo red or bromthymol blue dyes, Gram staining and cell shapes of isolated bacteria were examined according to Vincent (1976) and Somasegaran and Hoben (1994).

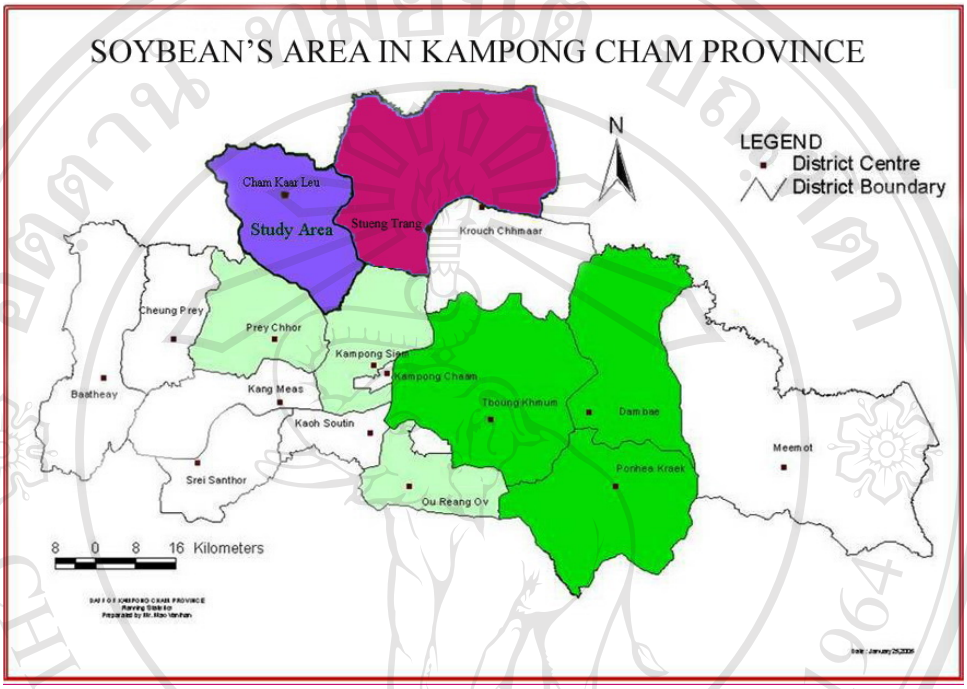

Fig. 2: Soybean cultivated field at Chamkaar Leu, Kampong Cham Province

3.3 Effectiveness testing of native root nodule bacterial isolates

This experiment was conducted in the controlled room with the average temperature about 25^{o}C and 16 hours per day for light period. Purified isolates of root nodule bacteria were used in this experimental step. Surface sterilized DT 84 soybean seeds which were pregerminated were sown in plastic cups containing sterile sand. One week after sowing, liquid culture (YMB) of each purified bacterial isolate was inoculated to soybean seedlings at the rate of 10^{9} cfu/cm/plant. Bacterial inoculated plants and uninoculated control ones were irrigated with N-free medium (Broughton and Dilworth, 1970; cited by Somasegaran and Hoben, 1994). The excess salts in the growth medium were washed out once a week using sterile distilled water.

The experimental design was completely randomized design (CRD) with 14 root nodule bacterial inoculated treatments and two control treatments without (U) and with 70ppm NO_3-N (N). Each treatment was replicated five times. At one month after inoculation, the plants were pulled out and the following data; nodule dry weight, root and shoot dry weight, and total plant N uptake were collected. To determine total N content in dried plant samples, the ground plant samples were firstly digested with the digestion block at the temperature of 330^{o}C (Novozamsky *et al.*, 1974 cited by Wallinga *et al.*, 1989). The total N in digested samples were determined by indolphenol blue method (Novozamsky *et al.*, 1974 cited by Wallinga *et al.*, 1989) N uptake of the whole plant was calculated according to this formula:

$$\text{N uptake} = \frac{\%\text{N} \times \text{DW (g) of the whole plant}}{100} \times 100$$

The data on nodule and total plant dry weight and N uptake of the whole plant of DT 84 soybean were statistically analyzed by F-test and the means were compared by least significant difference (LSD) at $P < 0.05$. Effectiveness of the tested root nodule bacterial isolates were considered from the data of N uptake of the whole plants using criteria of Ferreira and Margues (1992) for calculation of effectiveness indices (E-value) of root nodule bacterial isolates. The formula for calculation an index of effectiveness (E) was as follow:

$$E = \frac{I - U}{N - U} \times 100$$

I = the whole plant N uptake of inoculated treatment

N = the whole plant N uptake of NO_3 - applied treatment

U = the whole plant N uptake of inoculated control

Based on E-value the level of effectiveness of the tested root nodule bacterial isolates could be evaluated as follow: ineffective if E was less than 25%, moderately effective if E-value was 25-75%, highly effective if E was higher than 75%.

3.4 Pot Experiment

One early variety of Cambodian soybean DT 84 was used for pot experiment in order to evaluate its compatibility with selected endophytic actinomycetes and different soybean root nodule bacteria collected from Thailand and Cambodia. The experiment design was randomized complete block (RCB) with 5 replications and fourteen treatments as shown in Table 1. P_4 isolate of *Streptomyces* was tested previously by Thapanapongworakul (2003) as one of the effective selected endophytic actinomycetes which could be compatible with soybean and one of bradyrhizobial strain. This endophyte showed its antagonistic abilities against various plant disease (Thapanapongworakul, 2003). The selected root nodule bacterial isolates were the Cambodian representative root nodule bacteria from each studied sites in Cambodia.

In case of microbial inoculated treatment, peat inoculant of each tested microbes was used. Peat inoculant was prepared by mixing liquid culture of each tested microbe with autoclaved peat powder (autoclaving three times each at 121^{o}C for 1 hour) in plastic bag at the rate of 20 ml of each liquid culture per 20 g of peat. After mixing, the peat inoculant of each tested microbe was incubated at room temperature for 5 days in which the viable cell numbers of each inoculated microbe at least 10^8 cfu/g were obtained. The peat powder used for inoculum preparation had maximum water capacity about 276% (by weight basis). YMB was used for cultivation of each root nodule bacterial isolate while IMA_2 was used for endophytic actinomycetes. During liquid culture preparation, the flask containing liquid culture

of each tested microbe was shaked on rotary shaker until the culture with maximum turbidity (10^{8-9} cfu/ml) were obtained which took about 3-5 days. For inoculation, peat inoculant of each tested microbe was applied into the soil at the bottom of the hole prepared for seed sowing, then soybean seeds were placed on top of the applied inoculant and finally covered with the soil. The rate of inoculation were 10^6 cfu/seed for each nodule bacteria isolate and 10^6 cfu/seed for endophytic actinomycetes.

An open field pot experiment was conducted during May 07 to 22 July, 2009 at Department of Agronomy, Faculty of Agriculture, Chiang Mai University. The soil was collected from Meae Hae Research and training center, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand (18.48^{o}N, 98.59^{o}E). The soil was clay loam soil with pH 7.6 and contained 40-100 mg/kg of available P, 87 mg/kg of exchangeable K, 1447 mg/kg of exchangeable Ca and 101 mg/kg of exchangeable Mg at the time of sowing. During experimental period 07 May to 22 July 2009. The soybean plants were grown in plastic pots for 3 sets of growing stages such as V_6 stage, $R_{3.5}$ stage and maturing stage. The soil was sterilized at temperature about 121^{o}C for 1 hour by autoclaving before using for pot experiment in order to eliminate native population of root nodule bacteria. Each plastic pot was filled with 3 kg of soil per pot (8 inches in diameters) for V_6 and 12 kg of soil per pot (10-12 inches in diameters) for $R_{3.5}$ stage and maturing stage respectively. Ordinary type water was used for irrigation through out crop cultivation. Seven days after seed germination, the plants were thinned to get 3 plants per pot.

Table 1. Treatments used in pot experiment for each soybean variety

Treatment	Detail	Treatment code
1	Uninoculated control	U
2	Single inoculation with P_4 selected endophytic Actinomycetes *(Streptomyces sp.)*	EA
3	Single inoculation with CD_2P, selected Cambodia root nodule bacterial isolate	CD_2P
4	Single inoculation with CL_4HK, selected Cambodia root nodule bacterial isolate	CL_4HK
5	Single inoculation with CL_3B, selected Cambodia root nodule bacterial isolate	CL_3B
6	Single inoculation with CD_1YD, selected Cambodia root nodule bacterial isolate	CD_1YD
7	Single inoculation with Th7, Thailand standard Bradyrhizobium	Th7
8	Dual inoculation of EA and CD_2P	EA + CD_2P
9	Dual inoculation of EA and CL_4HK	EA + CL_4HK
10	Dual inoculation of EA and CL_3B	EA + CL_3B
11	Dual inoculation of EA and CD_1YD	EA + CD_1YD
12	Dual inoculation of EA and Th7	EA + Th7

CD_1 = Chumkaar Andong site 1, CD_2 = Chumkaar Andong site 2

CL_3 = Chumkaar Leu site 3, CL_4 = Chumkaar Leu site 4

P = purple flower soybean variety HK = hung kry soybean variety

B = B3039 soybean variety YD = yellow dark flower soybean variety

Since the sterile soil contained sufficient amount of available P, exchangeable K and exchangeable Ca and Mg, thus no P and K fertilizers were applied. Fipronil insecticide was sprayed at the beginning of soybean growth to prevent damaged by bean fly.

At V_6 (32 DAS) and $R_{3.5}$ (52 DAS) stages, soybean plants were harvested and separated into shoots, roots and nodules. Great care was taken to recover all roots and nodules by sieving the soil through a 1 mm sieve. At $R_{3.5}$ stage, root bleeding sap samples of the plants from each pot were collected. To collect the root bleeding sap samples the shoot below or up on the first node close to ground level of each plant was cut with a very sharp blade or secateurs.

The silicon or latex rubber tubing sleeve, 2-4 cm long with the internal diameter slightly smaller than the stem was placed over the exposed root stump.

Fig. 3: Bleeding sap samples of the plants from each of the plants

Root bleeding sap of soybean plants from each pot (Fig. 3) were collected from the tubing sleeve reservoir using a Pasteur pipette or syringe. Sap samples were collection by mixing with an equal volume of ethanol in the collection tube and then kept on ice until frozen at -15^{o} C for long term storage (Peoples *et al.*, 1989). The above ground parts and roots of the plants from each pot were dried by the oven at 70^{o} C for at least 72 hours for dry weight determination.

The collected dried shoot of soybean samples at $R_{3.5}$ stage were ground with mill for total N analysis (Novozamsky *et al.*, 1974; Novozamsky *et al.*, 1984 cited by Wallinga *et al.*, 1989). The root bleeding sap samples were analysis for amino-N (Yemm and Cooking (1955), cited by Herride, 1984), NO_3-N (Catado *et al.*, 1975) and ureide-N (Young and Conway, 1942).

Relative ureide index (RUI) of root bleeding sap was calculated according to (Peoples *et al.*, 1988).

$$\text{Relative ureide index (\%)} = \frac{4 \times \text{ureide}}{4 \times \text{ureide} + \text{amino acid} + \text{nitrate}} \times 100$$

Percentage of seasonal fixed N was calculated according to Herridge and Peoples (2002).

$$y = 4.8 + 0.83x \quad \text{(At early pod filling stage)}$$

Where: y = relative ureide index (%)

x = nitrogen derive from air (%)

The total N accumulation or N uptake of the shoot was calculated according to this formula:

$$\text{N uptake (g) of plant (g N/plant)} = \frac{\%N\ (g) \times \text{Dry Wt. (g)}}{100}$$

At 82 DAS, seeds yields of soybean plants from each pot were harvested. At this stage, the collected data were number of pods/plant and seed yield.

The data on nodule dry weight, root dry weight and shoot dry weight, number of pods/plant and seed weight/plant, RUI, percentage and a mount of seasonal N derive from N fixation, total N uptake per plant were statistically analyzed by F-test and the treatment means were compared by using least significant difference, (LSD = 0.05) (Gomez and Gomez, 1984).