CHAPTER III

MATERIALS AND METHODS

3.1 Collection of Root Nodule Bacterial Isolates from Myanmar Soil

Three soybean cultivated fields of the farmers in Myanmar were selected (Figure 1) as the target sites for collection of native root nodule bacteria from soils.

Figure 1. Three collection sites of soybean cultivated fields of the farmers in Myanmar

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The selected sites were (1) Naung Cho in Shan State, Upper Myanmar (Figure2), (2) Bagan in Mandalay Division, Middle Myanmar (Figure 3), Hleguu in Yangon Division, Lower Myanmar (Figure 4). The soils were used for isolation of root nodule bacteria because in March 2008 in which the field survey was conducted it was not possible to collect root nodule. One composite surface soil surface soil sample from each site was collected. The soil in each composite sample was mixed well and kept in plastic bag under room temperature without drying before isolation.

Figure 2. Soil collecting site from Naung Cho in Shan State, Upper Myanmar

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Figure 3. Soil collecting site from Bagan in Mandalay Division, Middle Myanmar

Figure 4. Soil collecting site from Hleguu in Yangon Division, Lower Myanmar

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3.2 Isolation of root nodule bacteria from Myanmar soils

The local soybean variety used by the owner of selected soybean field at Shan State was used as the trap host for isolation of root nodule bacteria from selected

Myanmar Soils. The seeds were surface-sterilized by soaking in 95% ethyl alcohol for 30 seconds and 5% sodium hypochloride solution for one minute and then rinsed with the sterilized distilled water three times. The surface sterilized seeds were germinated in sterile Petri-dish containing moist tissue paper. The germinated seeds with the root about 1cm long were aseptically transferred to 250ml glass bottle containing autoclaved sand and each of original soil from Myanmar.

There were two plants per bottle. All bottles were kept in screen house. During soybean cultivation for one month, the plants were irrigated through the cotton wig connected between the top part containing the mixture of sterile sand and soil, and the bottom part containing sterile N free solution. After seed sowing for one month, soybean plants in each bottle were carefully pulled out from the mixture of sand and original soil for nodule collection. The collected nodules were washed with running tap water to remove the mixture of sand and soil. The collected nodules of soybean were surface-sterilized by soaking in 95% alcohal for 10 seconds, follow by chlorox 3% for 2 minutes and then rinsed with the sterilized water 6 times. After surface-sterilization, each nodule was broken down by forceps and the bacteria in the nodule were streaked on YMA (yeast mannitol congo red agar) for 7 days. The typical colonies of root nodule bacteria on YMA were selected for purification (Weaver et al., 1994).

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3.3 Effectiveness testing of selected Myanmar root nodule bacterial isolates

The typical purified root nodule bacterial isolates from Myanmar were tested for their effectiveness on dry weight improvement of the whole plant of three soybean varieties, local soybean variety from Shan States, SJ 5 and one local soybean variety

from Cambodia. Method of seed sterilization and seed preparation used in this test were the same as those mention in 3.1. The test was conducted in the controlled room with 16 hours under light per day and at the temperature of 25ºC.

Soybean plants were grown in autoclaved sand in plastic cup using one plant per cup. N-free nutrient solution (Broughton and Dillworth, (1970) cited by Somasegaran and Hoben, 1984) was used throughout the growing period of one month. Leaching out the excess salt from the sand was done one time per week by using sterile water. In the effectiveness testing for each soybean variety, the experimental design was completely randomized design with 4 replications and 6 treatments as follows:

Treatment 1 uninoculation control,

Treatment 2 inoculation with USDA 110, the standard *Bradyrhizobium* strain,

Treatment 3 inoculation with THA 7, standard *Bradyrhizobium* strain from Thailand,

Treatment 4 inoculation with selected endophytic actinomycetes (*Streptomyces sp.*),

Treatment 5-6 inoculation with MA and MB, Myanmar root nodule bacterial isolates respectively.

The selected endophytic actinomycete was included to get preliminary data on the compatibility between this selected microbe and each soybean variety.

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3.4 Pot Experiments

Three recommended soybean varieties from Myanmar (Hintada), Thailand (SJ 5) and Cambodia (DT 84) were used for pot experiments in order to evaluate effects of endophytic actinomycetes and Bradyrhizobia on growth, nodulation,

nitrogen fixation and seed yield of different soybean varieties. One soybean variety was used for each pot experiment. In each pot trail, the experimental was laid out in a randomized complete block design (RCB) with 3 replications and 12 treatments as indicated in Table 1.

Soybean seeds of each variety were surface sterilized in 70% aqueous ethanol for 3 minutes followed 2 minutes in sodium hypochlorite solution (50 g/L) and then washed more than 6 times in sterile distilled water. The seeds were grown in peat moss media under controlled condition and irrigated with sterile distilled water to avoid possible contamination of the seedlings with Bradyrhizobia and endophytic actinomycetes from the other sources. After true first pair of leaves emerged, the plants were transplanted to the soil in the pots using three plants per pot.

The pot trails were conducted outdoor during 29 November 2008 to 15 March 2009 at Department of Agronomy, Faculty of Agriculture, Chiang Mai University. The clay loam soil collected from Mae Hae Research and Training Center, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand (18.48 N, 98.59º E) was used with pH 7.6. The soil was autoclaved at 121º C for 1 hour before using in each pot trial. After autoclaving, the soil had pH 7.6, 44 ppm N as NH4^{+}N, 15 ppm N as NO3^{-}N, 40 mg/kg of available P, 87 mg/kg of exchangeable K, 1447 mg/kg of exchangeable Ca and 101 mg/kg as exchangeable Mg at the time of sowing. The soybean plants were grown in plastic pots for 3 sets of growing stages such as V6 stage, R 3.5 stage and harvest stage.

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

Treatment	Detail	Treatment code
1	Uninoculated control	U
2	Single inoculation with selected endophytic actinomycetes (*Streptomyces sp.)*	EA
3	Single inoculation with MA, selected Myanmar bradyrhizobial isolate	MA
4	Single inoculation with MB, selected Myanmar bradyrhizobial isolate	MB
5	Single inoculation with USDA 110, standard bradyrhizobial isolate	USDA 110
6	Single inoculation with THA 7, Thailand standard bradyrhizobial isolate	THA 7
7	N-applied treatment using urea at the rate of 6 kg N /rai	N
8	Inoculation of EA plus 6 kg N /rai	EA + N
9	Dual inoculation of EA and MA	EA + MA
10	Dual inoculation of EA and MB	EA + MB
11	Dual inoculation of EA and USDA 110	EA + USDA 110
12	Dual inoculation of EA and THA 7	EA + THA 7

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The pots filled with 3 kg of sterile soil were used for data collection at V6 stage, while those filled with 7 and 12 kg of soil per pot were used for R 3.5 stage and at maturing stage respectively. Ordinary tap water was used for irrigation through out crop cultivation.

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. N- fertilizer

was applied by equally split application 2 times, at 12 days after sowing and at R3.5 stage. Fipronil insecticide was sprayed at the beginning of soybean growth to prevent damaged by bean fly. Mancozeb fungicide was also sprayed after R3.5 stage for disease control.

At V6 and R3.5 stages, 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 R3.5 stage, root bleeding sap samples of the plants from each of the plants from each pot were collected. To collect the root bleeding sap samples the shoot below the first node close to ground level of each plant was cut with a very sharp blade or pair secateurs. The silicon or latex rubber tubing sleeve, 2-4 cm long with and internal diameter slightly smaller than the stem was placed over the exposed root stump.

The sap exuding under root- pressure can be easily collected from the tubing sleeve reservoir using a Pasteur pipette or syringe. Sap samples were kept on ice until frozen at -15ºC for long term storage, or stabilized immediately after collection by mixing with an equal volume of ethanol in the collection tube if ice is unavailable (Peoples *et al*., 1989).

The above ground parts and roots of the plants from each pot were dried by the oven at 70ºC for at least 72 hours for dry weight determination. The collected dried shoot of soybean samples at R3.5 stage were ground with mill for total N analysis (Novozamsky *et al*., 1974). The root bleeding sap samples were analyzed for amino-N (Yemm and Cooking, 1955), $NO3^{-}$N (Cataldo *et al*., 1975) and ureide-N (Young and Conway, 1942).

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

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

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

$y = 4.8 + 0.83x$ (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:

$$\text{N uptake (g) of plant or seed} = \frac{\text{\% N (g) x Dry Wt. (g)}}{100}$$

The data on nodule dry weight, root dry weight and shoot dry weight, number of pods / plant, number of seeds / pod and seed weight/ plant, RUI, percentage and amount of seasonal N derived 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 (Gomez and Gomez, 1984).

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