CHAPTER III

MATERIALS AND METHODS

3.1 Analysis of soil samples

Soil samples were collected from three districts : Chart Trakarn (3 sub-districts), Bang Rakam (8 sub-districts) and Prom Piram (4 sub-districts) in Phitsanulok Province and sent to Department of Microbiology, Faculty of Science, Chulalongkorn University, by Dr. Wipa Homhaul. One kilogram of soil from each sub-district was sent to the office of Department of Agriculture, Bangkhen district, Bangkok, for analyses of soil moisture content, water holding capacity, available P, available K, organic matter, Na, Ca, Mg, Fe, Mn, Zn, Cu, S, Cl. Soil pH was determined at the Department of Microbiology, Faculty of Science, Chulalongkorn University, as follows : Twenty ml of 0.01 M $CaCl_2$ were added to ten grams of each soil sample in a beaker and stirred with magnetic stirrer for 30 mins. The suspension was left 30 mins for soil particles to settle. pH of the clear upper solution was determined by pH meter (Mettler).

3.2 Isolation of bacteria from root nodules of soybeans grown in three districts in Phitsanulok province

Soybean seeds of seven cultivars (ST1, ST2, ST3, SJ4, SJ5, CM2 and CM60) were surface- sterilized by rinsing in 95% ethanol for 10 seconds to remove waxy materials. The seeds were surface- sterilized with 5% H_2O_2 for 3-5 mins as described by Somasegaren and Hoben (1994). The sterilized seeds were imbibed in sterilized de-ionized water at 4 ^{0}C for 4 hours. Seeds were aseptically placed on 0.75% seeding agar (0.75%), incubated in the dark at 25 °C for 36 h. Germinating seeds were put in pots containing soils from three districts of Bang Rakum, Prom Piram and Chat Trakarn, Phitsanulok Province. Five germinating seeds were placed in each pot . The germinating seeds were watered with nitrogen-free nutrient solution, pH 6.8. Composition of nitrogen-free medium was given in Appendix A. The plants were thinned to two plants per pot after 14-day growth . Plants were grown for 28 days at 28 °C in a greenhouse before root nodules were collected. Five large nodules (diameters ranging from 0.3-0.8 cm).

were selected from the two plants per pot. Bacteria from each nodule were plated on yeast extract mannitol agar (YMA) with 25 µg.ml-1 congo red. A total of five colonies was selected for each combination of soil and soybean cultivar. One out of the five colonies from each combination of soil and soybean cultivar was chosen for PCR fingerprinting. Each pure culture was kept in YMA slants at 4 °C.

3.3 Growth of isolates in yeast extract mannitol medium (YMB)

Cell culture was activated by growing on fresh YMA slants at 25 °C. 1 loop of activated cells was put into 50 ml of YMB. Cells were grown until mid-log phase at 30°C, 200 rpm for 2-7 days. Composition of YMB was given in Appendix A. Growth was followed by optical density readings at 660 nanometer.

3.4 RAPD-PCR fingerprinting

3.4.1 Isolation of chromosomal DNA

Cells of each isolate were activated by culturing on yeast extract mannitol (YMA) slants with 25 µg.ml-1 congo red at 30°C . One loop of each activated isolate was inoculated into 50 ml yeast extract mannitol broth (YMB). The culture was grown at 200 rpm, 30°C until mid log phase. Cells were harvested by centrifuging one ml cell suspension at 8,000 rpm, 4°C for 5 minutes. 80 µl 2.5 $mg.ml^{-1}$ lysozyme was added to the cell pellet, mixed thoroughly, and incubated in a 37°C water bath for 1 h before 4 cycles of freezing at –20°C for 5 minutes and thawing at 80°C for 5 minutes. 250 µl DNAzol®(Invitrogen) was added to the solution which was gently mixed by inverting the eppendorf tubes. The mixture was centrifuged at 10,000 rpm, 4°C for 5 minutes. The supernatant was transferred to a fresh eppendorf tube. 500 µl ice-cold ethanol was added to the mixture which was gently mixed by inverting the tube before centrifugation at 12,000 rpm, 4°C for 10 minutes. The precipitate was washed with 70% ice-cold ethanol and air dried in a laminar flow hood. Twenty µl high-purity distilled water was added to dissolve the nucleic acid precipitate at room temperature . Quantity of isolated chromosomal DNA was determined by absorbance at 260 nm and quality of the isolated chromosomal DNA was checked by OD_{260}/OD_{280} ratios and 0.8% agarose gel electrophoresis by standard methods (Sambrook et al, 1989).

3.4.2 RAPD-PCR fingerprinting

Sequences of RPO1 and CRL-7 were as reported by Richardson et al. (1995) and Mathis & McMillin (1996) as follows : RPO1 : 5'AATTTTCAAGCGTCGTGCCA3' ; CRL-7 : 5'GCCCGCCGCC3'.

All primers were synthesized by Invitrogen™, Hong Kong. To obtain reproducibility all RAPD-PCR fingerprinting experiments were repeated at least twice. When *Taq* polymerase from Invitrogen™ and dNTP from Promega were used, PCR was performed in the following mixture (A). When *Taq* polymerase and dNTP (iNtroN Biotechnology) were used, PCR was performed in the following mixture(B).

Mixture (A)		Mixture (B)		Program		
10x PCR buffer	2.5 µl	10x PCR buffer	2.0 µl			
50 mM $MgCl_2$	0.8 µl	dNTPs mixture	2.0 µl	95 °C	15 seconds	5 cycles
10 mM dNTPs	0.5 µl	(2.5mM each)		55 °C	30 seconds	
10 pmol primer	5.0 µl	Primer1	5-10 pmol	72 °C	90 seconds	
DNA template	1.0 µl	Primer2	5-10 pmol	95 °C	15 seconds	25 cycles
(60-100 ng)		DNA template	1ng-1µg	60 °C	30 seconds	
Taq polymerase	0.2 µl	*i-Taq*™ polymerase	0.2 µl	72 °C	90 seconds	
($5U.\mu l^{-1}$)		($5U.\mu l^{-1}$)		72 °C	10 minutes	
High quality double distilled water	15.0 µl	High quality double distilled water up to	20.0 µl			
Total	25.0 µl	Total	20.0 µl			

PCR products were separated by 1.25 % agarose gel electrophoresis and stained in 0.5 $\mu g/ml^{-1}$ Ethidium Bromide by standard methods (Sambrook et al., 1989). RAPD-PCR fingerprints were viewed and photographed on a UV transilluminator (Bio-rad).

3.5 Multiplex PCR

3.5.1 Design of primers

Sequences of *nodD1* and *nodY* were downloaded from National Center for Biotechnology Information (NCBI) website. Alignments of the sequences were obtained by the bioedit program (http:// (http://www.mbio.ncsu.edu) , conserved sequences were selected for use as forward and reverse primers in multiplex PCR , taking into consideration similarity in Tm and %GC and that the primer sequences did not self-anneal. Formula for determination of Tm was as described by Pastorino et al. (2003) as follows :

T_m was calculated by $T_m = 63.3 + 0.41 \times \%GC - 500/length$ (Pastorino et al., 2003)

3.5.2 Optimization of multiplex PCR reactions.

PCR mixture was described in section 3.4 DNA of fast-growers and slow-growers were used as well as DNAs of *Agrobacterium tumefacians* TISTR 507, *Xanthomonas campestris*

Mixture			Program		
10x PCR buffer	2.5	µl	95°C	15 seconds	5 cycles
50 mM $MgCl_2$	0.8	µl	50°C	30 seconds	
10 mM dNTPs	0.5	µl	72°C	90 seconds	
10 pmol *nodD1*F	1.25	µl	95°C	15 seconds	25 cycles
10 pmol *nodD1*R	1.25	µl	55°C	30 seconds	
10 pmol *nodY*F	1.25	µl	72°C	90 seconds	
10 pmol *nodY*	1.25	µl	72°C	10 minutes	
DNA template (200 ng)	1.0	µl			
High quality double distilled water	15.0	µl			
Total	25.0	µl			