Chapter 3

Materials and Methods

3.1 Bacterial isolates and soybean cultivars

Nineteen *B. japonicum* isolates used in the following experiments were isolated by Mr. Suwat Saengkerdsub (1999) from acidic soil samples taken from five provinces in Thailand as indicated in Table 3.1. The isolates were kept in yeast extract mannitol agar slants at 4 °C. The composition of YMA was as described in Appendix A.

TABLE 1. Soil sample collection sites (Saengkerdsub, 1999).

Place	District	Province	Soil pH	Isolates
Nern Mahatsajan	Kao Kaw	Petchaboon	5.25	S76, S78, S162,S185, S187
Tad Mork Falls	Muang	Petchaboon	4.87	S180, S182, S184
-	Potale	Pijit	4.54	S198, S205
Ta-Saeng	-	Nakorn Sawan	5.73	S192
-	Kanuworaraksaburi	Kampaeng Pet	5.82	S202
-	Meenburi	Bangkok	4.78	S8, S18, S40, S42,S57,S58, S74

The following soybean cultivars were used : *Glycine max* Chiangmai 2 (CM 2), Chiangmai 60 (CM 60) and Sukhothai 2 (ST 2). Soybean seeds were obtained from Department of Agriculture, Bangkhen district, Bangkok.

3.2 RAPD-PCR fingerprinting of 19 *B. japonicum* isolates

3.2.1 Isolation of chromosomal DNA

Cells of each isolate were activated by culturing in yeast extract mannitol agar slants (YMA) at 30°C for 2 days. One loop of each activated isolate was inoculated into 50 ml yeast extract mannitol broth (YMB). The composition of YMB was as described in Appendix A. The culture was grown at 200 rpm, 30°C until mid log phase. Cells were

harvested by centrifuging one ml cell suspension at 5,000 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. One volume of 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 10,000 rpm, 4°C for 15 minutes. The precipitate was washed with 70% ice-cold ethanol and air dried in a laminar flow hood. Thirty µl high-purity distilled water was added to dissolve the nucleic acid precipitate at room temperature for 1 day. Quantity of isolated DNA was determined by absorbance at 260 nm and quality of the isolated chromosomal DNA was checked by 0.8% agarose gel electrophoresis by standard methods (Sambrook & Russell, 2001).

3.2.2 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 Life Technologies, USA. To obtain reproducibility all RAPD-PCR fingerprinting experiments were repeated at least twice.

RPO1 primer was used in RAPD-PCR fingerprinting in the following mixture:

Mixture			Program		
10x PCR buffer	2.5	µl	95°C	15 seconds	5 cycles
50 mM $MgCl_2$	0.8	µl	55°C	30 seconds	
10 mM dNTPs	0.5	µl	72°C	90 seconds	
10 µM primer	5.0	µl	95°C	15 seconds	25 cycles
DNA template (60-100 ng)	1.0	µl	60°C	30 seconds	
Taq polymerase ($5U.\mu l^{-1}$)	0.2	µl	72°C	90 seconds	
High quality double distilled water	15.0	µl	72°C	10 minutes	
Total	25.0	µl			

CRL-7 primer was used in RAPD-PCR fingerprinting in the following mixture :

Mixture			Program		
10x PCR buffer	5.0	µl	95 °C	15 seconds	5 cycles
50 mM $MgCl_2$	1.5	µl	55 °C	30 seconds	
10 mM dNTPs	1.0	µl	72 °C	90 seconds	
10 µM primer	5.0	µl	95 °C	15 seconds	25 cycles
DNA template (60-100 ng)	2.0	µl	60 °C	30 seconds	
Taq polymerase ($5U.\mu l^{-1}$)	0.25	µl	72 °C	90 seconds	
High quality double distilled water	35.25	µl	72 °C	10 minutes	
Total	50.00	µl			

PCR products were separated by 1.25 % agarose gel electrophoresis by standard method (Sambrook & Russell, 2001). RAPD-PCR fingerprints were viewed and photographed on a UV transilluminator (Bio-rad).

3.3 Growth of *B. japonicum* in unbuffered and buffered medium

One loop of activated *B. japonicum* S76 or S78 or S162 was inoculated into 50 ml of YMB, pH 7.0 and grown to mid log phase to be used as seed culture. Five ml of each seed culture was inoculated into 45 ml of unbuffered or buffered YMB, pH 4.0-9.0. 30 mM NEDA, 30mM MES, 30mM HEPES and 30 mM AMPSO were used to buffer pH 4.0-5.0 (NEDA), pH 6.0 (MES), pH 7.0-8.0 (HEPES) and pH 9.0 (AMPSO). Growth was monitored by measuring turbidity at wavelength 660 nanometer. Contamination was checked and final pHs were measured at the end of the experiments.

3.4 Extraction of intracellular proteins

3.4.1 Protein profiles of cells grown in unbuffered medium

Seed culture was prepared by inoculating one loop of activated *B. japonicum* into 50 ml of YMB medium, pH 7.0. The culture was grown at 200 rpm, 30°C until mid log phase. Five ml of each seed culture were inoculated into a set of 45 ml YMB medium with initial pH ranges from 4.0-8.0. Cultures were grown at 200 rpm, 30°C until mid log phase as determined by turbidity measurement at wavelength 660 nanometer.

Intracellular proteins were extracted by harvesting cells at 12,000 rpm, 15 min at 4°C. Cell pellet was washed twice with extraction buffer (0.5 M Tris HCl, pH 7.0). Two to three volumes of sterilized glass beads (Sigma G-9143) were added to the cell pellet suspended in 80 µl extraction buffer, vortexed at top speed for 40 seconds, left on ice then the vortexing was repeated 9 more times with tubes on ice after each vortexing. Contents were centrifuged at 12,000 rpm, 40 minutes at 4°C. Concentrations of soluble proteins in the supernatant were determined by the Bradford method (Bradford, 1976) using the protein dye assay (BIO-RAD) with Bovine Serum Albumin as the standard. Soluble proteins were separated by SDS-PAGE as described by Laemmli (1970) with 50 µg protein per well. Proteins were stained by Silver stain kit (BIO-RAD) according to the manufacturer 's instruction.

3.4.2 Protein profiles of cells grown in buffered medium

The experimental procedure was the same as that described in section 3.4.1 except 5 ml of seed culture were inoculated into 45 ml of buffered YMB, pH 5.5, 6.0, 7.0 and 8.0 with 30 mM MES as the buffer for pH 5.5-6.0 and 30mM HEPES as the buffer for pH 7.0-8.0.

3.4.3 Effects of changes in medium pH on intracellular protein profiles

Seed culture was obtained by growing cells as described in section 3.4.1 except pH of the YMB medium was 5.5 with 30 mM MES as the buffer. Cells were grown until mid log phase before 5 ml of cell suspension were aseptically harvested, washed and resuspended with 5 ml sterilized distilled water before transferring to 45 ml of YMB medium pH 6.0, 6.5 and 7.0 containing the following buffers respectively, 30 mM MES, 30 mM MES and 30 mM HEPES. Growth was determined by measuring absorbance at 660 nm. Cells at 0, 12 h, 18h, 24h, 3 days and 5 days after the pH shifts were harvested and soluble proteins extracted and separated by SDS-PAGE as described in section 3.4.1.

3.5 Transblotting of intracellular proteins

Polypeptide bands in SDS-PAGE gels obtained from section 3.4.3 with 100 µg protein loading per well were transferred to Millipore Immobilon-P transfer membrane

which is polyvinylidene fluoride (PVDF) according to the manufacturer's instruction. Polypeptide bands were visualized by staining the membrane in 0.5% Ponceau-S and 1% acetic acid. A 53 kDa band was cut and sent to the Bioservice Unit, National Center for Biotechnology and Genetic Engineering, Bangkok, for N-terminal amino acid sequencing. Sequence of the first ten amino acids was compared with available protein sequences deposited in Swissprot using the NCBI BLAST program.

3.6 Determination of plant and nodule dry weights

3.6.1 Seed surface-sterilization and germination

Soybean seeds cultivar CM2, CM60 and ST2 were surface-sterilized as described by Somasegaran and Hoben (1994). Seeds were placed in an Erlenmeyer flask, (wide-mouthed and previously sterilized by autoclaving). The mouth of the flask was covered with half of a sterilized petridish. The seeds took up about 25% of the volume of the flask. The petridish cover was kept in place throughout the operation. The seeds were rinsed in 95% ethanol for 10 seconds to remove waxy materials and trapped air, after that ethanol was drained off. 5% hydrogen peroxide solution was added in sufficient volume to immerse the seeds completely. The content was swirled gently to bring the seeds and 5% hydrogen peroxide into contact. After 3-5 minutes, the sterilizing liquid was drained off and the seeds rinsed with six changes of sterilized water. Aseptic procedures were observed throughout the rinsing. After the sixth rinse, the seeds were submerged in water and left in the refrigerator for 4 hours for seed imbibition. After 4 hours, the seeds were rinsed with two changes of water and plated on 0.75% (w/v) water agar in petridishes. About 20-50 seeds were placed per plate and were incubated at 25 °C in the dark for 2 days.

3.6.2 Growth of soybean plants in Leonard jars

Preparation of Leonard jars was as described by Somasegaran & Hoben (1994). Three germinating seeds were placed in each Leonard jar. Nitrogen-free medium, either pH 5.0 or pH 6.8, was used to water the subsequent seedlings and plants for 28 days. Plants were thinned to two plants per Leonard jar after growth for 2 weeks. Plant dry weight was obtained by cutting the shoot portions of the two plants at the

cotyledon scar for drying at 70 °C for 72 h. Nodule dry weight was obtained by weighing all dried nodules of the two plants grown in each Leonard jar. Average dry weight per plant and nodule dry weight per plant were reported. Statistic analysis was obtained by Duncan's Multiple Range Test (Steel & Torrie, 1980).

higher plant dry weight. *B. japonicum* S76 and S162 were found to poorly nodulate all the three soybean cultivars at pH 5.0 resulting in the same plant dry weights as those of the corresponding negative controls.

Figure 4.4 indicated that when nitrogen-free medium pH 6.8 was used, inoculation of *Glycine max* cv CM 2 and CM 60 with *B. japonicum* S76 or S78 yielded the same level of average soybean dry weight as that of the positive control for *Glycine max* cv CM 2. Inoculating *Glycine max* cv ST 2 with *B. japonicum* S76 and S78 yielded higher soybean dry weight than that of the positive control while *B. japonicum* S162 was not found to increase soybean cv ST 2 dry weight when compared with the positive control. The results indicated that *B. japonicum* S162 in conjunction with the use of pH 6.8 nitrogen-free medium resulted in the highest average soybean dry weight for *Glycine max* cv CM 60. Since Figure 4.5 indicated that *B. japonicum* S78 and S162 yielded the same level of nodule dry weight when *Glycine max* cv CM 60 was watered with pH 6.8 nitrogen-free medium, the higher plant dry weight when *B. japonicum* S162 was the inoculant for *Glycine max* cv CM 60 might result from higher nitrogen-fixing ability of *B. japonicum* S162 at pH 6.8.

Duncan's Multiple Range test as indicated in Table 4.2 confirmed the findings that when nitrogen-free medium pH 5.0 was used, a combination of *B. japonicum* S78 and *Glycine max* cv. CM 60 yielded the most average plant dry weight (1.44 $g.plant^{-1}$) which was in the same range as that of the positive control (1.61 $g.plant^{-1}$). Table 4.4 showed that when nitrogen-free medium pH 6.8 was used, *B. japonicum* S162 was found to yield the most average plant dry weight when *Glycine max* cv. CM 60 was used (1.96 $g.plant^{-1}$) and the average dry weight obtained was found in the highest statistically significant level. The average plant dry weight obtained was statistically higher than that of the corresponding positive control (1.01 $g.plant^{-1}$) as shown in Table 4.4. The overall results as shown in Tables 4.2-4.5 indicated that at both pHs *Glycine max* cv. CM 60 yielded the most average plant dry weight and that *B. japonicum* S78 was found to be a good nitrogen-fixer for *Glycine max* cv CM 60 at pH 5.0 while S162 was found to be a good nitrogen-fixer for *Glycine max* cv CM 60 at pH 6.8.