CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial strain

Klebsiella strain R15 was isolated from the rhizosphere of rice grown in Thailand at Rangsit Rice Experimental Station by Harinasut, 1981.

2.2 Rice seeds

Breeder seeds of *Oryza sativa* L. cv. NMS4, KDML105 and RD7 were obtained from the Department of Rice Research, Ministry of Agriculture and Cooperative, Thailand.

2.3 Media

2.3.1 Luria broth medium (LB) (Luria et al., 1960)

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
Sodium chloride	10.0 g/l

Adjust pH to 7.0 with 1.0 N NaOH and 15 g/l agar was added for solid medium.

2.3.2 Nitrogen-free medium for bacteria (NF), (Döbereiner, 1977)

K_2HPO_4	0.05 g/l
KH_2PO_4	0.15 g/l
$CaCl_2 \cdot 2H_2O$	0.01 g/l

$MgSO_4-7H_2O$	0.02	g/l
$NaMoO_4 \cdot 2H_2O$	0.002	g/l
$FeCl_3$	0.01	g/l
$NaHCO_3$	0.01	g/l
Glucose	20.0	g/l

Adjust pH to 7.0 with 0.1 NaOH or 0.1 HCl

2.3.3 Nitrogen-Free Weaver medium for rice seedlings (Weaver et al., 1975)

Solution A

$ZnSO_4 \cdot 7H_2O$	0.43	mg/l
$MnSO_4 \cdot H_2O$	1.30	mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.75	mg/l
H_3BO_3	2.80	mg/l
$CuSO_4 \cdot 7H_2O$	0.026	mg/l
$CuSo_4 \cdot 7H_2O$	0.07	mg/l

Solution B

$MgSO_4 \cdot 7H_2O$	100	mg/l
$CaCl_2 \cdot 2H_2O$	100	mg/l
$FeSO_4 \cdot 7H_2O$	22	mg/l
Sodium EDTA	20	mg/l
Solution A	20	ml

Solution C

Dipotassium hydrogen phosphate	0.9	g/l
Potassium dihydrogen phosphate	0.6	g/l

Working solution was prepared by mixing 50 ml of solution B and 15 ml solution C, then made up the final volume to 1 liter.

2.4 Bacterial culture

To activate the bacteria, *Klebsiella* R15 were grown in LB plate for 24 h at 30°C. A single colony was picked up and transferred to a 250-ml Erlenmayer flask containing 40 ml of LB broth, incubated at 30°C in a shaking water bath until early stationary phase (OD_{420} reached 0.8-0.9). Three percent inoculum was transferred to a 250 ml of nitrogen-free (NF) medium in a 1000-ml Erlenmayer flask and incubated at the same condition described previously. Cultures at mid-log phase were harvested by centrifugation at 7,000 x g, 4°C for 30 min, washed once in 0.85% NaCl solution and centrifuged. The pellet was resuspended in 0.85% NaCl to adjust the final concentration at 10^9-10^{10} cells/ml.

2.5 Plant growth and Inoculation of *Klebsiella* R15

Rice seeds were surface-steriled by consecutive treatment of 70% ethanol (30 sec), sterile distilled water, containing 2-5 drops of Tween-20 per 100 ml, followed by immersion in 40%, 30% and 20% Clorox (commercial bleach), containing 2-5 drops of Tween-20 per 100 ml, for 25, 20 and 15 min, respectively. After extensive washing with sterile distilled water, the grains were germinated on 0.6% semisolid agar plates, incubated in the dark at 25°C for 3 days. Uncontaminated germinated seeds were grown hydroponically in a growth chamber containing 1.5 liters of Nitrogen-Free Weaver medium or N_2 fixing activity. For acetylene reduction assay (ARA), three germinated seedlings were transferred to a 130 ml tube containing 6.0 ml of Nitrogen-Free Weaver medium. The chambers and tubes were maintained in a controlled environment (12/12 hours dark/light cycle with cool fluorescent lamps at a temperature of 25/28°C).

On day 7 after germination, each tube containing 3 seedlings were inoculated with 1.0×10^9 cells of *Klebsiella* R15 in 1 ml of 0.85%

NaCl. For control tube, seedlings were added with 1 ml of 0.85% NaCl. For rice seedlings in growth chamber, 50 ml of suspension bacteria were added and 50 ml of 0.85% NaCl were added to control plants.

2.6 Tissue extraction

One lot of 50 plants was taken at time intervals (7, 14, 21 and 28 days after germination). The plants were dissected into shoot and root fractions. Each fraction was homogenized separately in a precooled mortar and pestle, and extracted with 0.1 M phosphate buffer, pH 7.4 containing 0.15 M NaCl (PBS). The shoot or root homogenate was filtered through cheesecloth and centrifuged at full speed in a top-bench centrifuge (Hettich, Model EBA 33). The supernatant fraction was stored at -20°C until use.

2.7 Enzyme-linked immunosorbent assay, ELISA (Chaopongpang, 1989)

Immunoassay was used to quantitate the amount of rice lectin in crude extracts by comparing with purified lectin standard curve. For every microtiter plate, various concentrations of standard purified embryo lectin (1-50 µg/ml) were measured along with crude extracts using six replicates for every sample. All operations were carried on at 37°C in a moist chamber unless specified.

Each well of the polystyrene microtiter plate was coated with 100 µl of 0.2% ovalbumin in 0.1 M sodium carbonate buffer, pH 9.6. The plate was incubated at 4°C, overnight. After precoating with ovalbumin, the plate was emptied and washed three times with 200 µl of 0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl, 0.05% (v/v) Tween-20 and 0.02% sodium azide (PTN). This procedure was used in subsequent washing step. Appropriate dilution of rice crude extract or standard lectin in PBS was added (100 µl) to an individual well. After

incubation for 30 min, washing step was repeated. Then 100 µl of antilectin diluted 1:15,000 in PTN was added in each well. The plate was incubated for 45 min. After washing the second antibody of goat antirabbit IgG-alkaline phosphatase conjugate (Sigma) at the dilution of 1:1,500 in PTN was added (100 µl/well). After incubation for 45 min and washing. The enzymatic reaction was performed by adding 100 µl of 1 mg/ml p-nitrophenylphosphate (Sigma) in 0.05 M sodium carbonate buffer, pH 9.6 containing 0.02% $MgCl_2$. The reaction was stopped after 1 h of incubation, by adding 50 µl of 1 N NaOH in each well. The absorbance at 405 nm was read with a Titertek Multiscan Plate Reader (Flow laboratories). Blank wells where only PTN buffer were added instead of standard lectin or tissue extracts were used to zero the instrument.

2.8 Protein determination (Bradford, 1976)

One hundred microliters of each tissue extract was mixed with 1 ml of protein reagent (consisting of 100 mg Coomassie brilliant blue G-250, from Fluka, dissolved in 50 ml of 95% ethanol and 100 ml of 85% w/v phosphoric acid and made up to 1 liter with distilled water). The mixer was left at room temperature for 5-20 min. Absorbance at 595 mm was measured in a Spectronic 2000 (Baush & Lomb, Milton Roy Company). Bovine serum albumin (1 mg/ml) was used as standard reference concentration.

2.9 Nitrogenase activity

Nitrogenase activity was assayed by Acetylene Reduction Activity (ARA) as described by Burris (1974). One set of experiment designed to determine nitrogenase activity is composed at 3x10 replicative tubes of 1) free-living bacteria *Klebsiella* R15, 2) free-living rice seedling

and 3) rice seedlings in association with *Klebsiella* R15.

Each replicative tube (130 ml total volume) was closed with a serum stopper, and 10% of the air volume (13 ml) was removed and refilled with an equal volume of 99.9% acetylene (Ratanachoke, Engineering Ltd.). After bacterial inoculation, gas sample (300 µl) was withdrawn, at regular interval (48 h) and injected into a gas chromatography (Packard Model 437 A) equipped with a Flame-Ionization Detector, and a stainless steel column (length 2 m, diameter 1/8") packed with Parapak N to measure ethylene production. Standard ethylene (99.9%) (Thai Industrial Gas Ltd.) was diluted to proper concentration with air, and used as reference to estimate C_2H_4 produced

2.10 Total Nitrogen content (Hiller et al., 1948)

When rice seedlings (30 plants) were 21 days old, they were cut separately into roots and shoots. The two separated fractions were dried in oven at 70°C for 48 h. The dried samples were weighed and put into digestion tubes. Add about 0.6 g of the catalyst mixture (K_2SO_4:$CuSO_4 \cdot 5H_2O$:SeO 30:4:1) and 6 ml of concentrated H_2SO_4 per tube. Boil the sample gently in a digestion unit (Kjeldatherm, Gerhardt) for about 15 min until the digested solution has become clear. Cool down to room temperature and dilute with 10-15 ml distilled water before transferring to a distillation unit (BÜCHI, Model 315). Distillation was performed by adding 5 ml of 40 g/l H_3BO_3 solution and 5 ml of distilled water and a drop of methyl red-bromocresol green indicator in a receiving flask placed at the end of the condenser. Add about 20 ml of 40% NaOH to the digestion tube. Pass steam through the distillation apparatus until the total volume of solution in the receiving flask was about 150 ml. The distillate was then titrated with standardized 0.1 M H_2SO_4. Blank was carried out at the same condition as described above

but omitting the sample.

% Nitrogen content was calculated as follows:

% Nitrogen = $[\{(v_1-v_2)\ M \times 0.0140\}/W] \times 100$

where :

v_1 = ml of H_2SO_4 required for titration of contents of the receiving flask

v_2 = ml of H_2SO_4 required for titration of the blank

M = molarity of the H_2SO_4

W = weight of sample in grams

0.0140 = mmole mass of nitrogen

2.11 Immunoblot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970)[4]. Electrophoresis was performed in a 12% slab gel and 3% stacking gel layer (Midget, LKB) at a constant current (20 mA per gel). After electrophoresis, the separated protein bands were immediately transferred to nitrocellulose paper (LKB) in a transfer tank (LKB "Trans Blot" Model) according to Towbin 1979. The buffer used was 0.25 M tris, 0.129 M glycine, and 20% (v/v) methanol. The transfer was conducted at approximately 1.2 mA for 1 h. After transfer was completed, the membrane was placed in a clean plastic tray and incubated in the blocking solution (1% (w/v) BSA in 50 mM Tris-HCl buffer, pH 7.5 containing 0.15 M NaCl, TBS) with gently shaking, overnight. The membrane was washed 3 times (10 min each) with TBS-0.1% Tween-20 and then incubated with antilectin diluted 1:500 in blocking solution. After shaking for 1 h, washing step was performed. The second antibody, horseradish peroxidase anti-rabbit IgG (Zymed Laboratories Inc.), diluted 1:1,500 in blocking solution, was then added and again incubated

with gently shaking for 1 h, then followed by repeated washing step. The specific protein-antibody complex was visualized by adding 10 ml of 0.03% Diaminobenzidine, DAB (Zymed Laboratories Inc.) in TBS with addition of 10 µl of 30% H_2O_2. The reaction was stopped by washing membrane with several changes of distilled water.

2.12 Preparation of colloidal gold (Horisberger, 1985)

Colloidal gold particles 20 nm in diameter was prepared according to Horisberger (1985). Starting by adding 1 ml of 1% Tetrachloroauric acid ($HAuCl_4$) into 200 ml of tripled-distilled water in a clean, siliconized Erlenmayer flask and heated until boiling, then 4 ml of 1% trisodium citrate was added. The solution was stirred vigorously and boiled until the color developed to red or red violet. The gold sols obtained were cooled and kept at 4°C.

2.13 Preparation of colloidal gold-protein A complex (Horisberger, 1985)

In order to conjugate protein A to colloidal gold, adsorption isotherms were prepared to determine the minimum amount of protein and pH optimum required for stabilization of colloidal gold.

2.13.1 Optimal amount of protein A to stabilize colloidal gold

A serial dilution of protein A (Zymed Laboratories, Inc.), started from 2 mg/ml, was prepared in 100 µl volume in series of 5-ml tubes. A constant amount of gold sols (1.0 ml) was added into each protein A tube and mixed. The solution was allowed to stand for 5 min, then 0.1 ml of 10% NaCl solution was added and coagulation was judged visually. The change in color from red to faintly blue or violet indicates the unstabilized particles. The optimal amount of protein A was selected for the further experiment.

2.13.2 Optimal pH for adsorption

The pH-variable adsorption isotherms were prepared in the same manner except that each tube containing gold sols, which has already been adjusted the pH with 1.0 M H_3PO_4 and 0.2 M K_2CO_3 to various pH, ranging from 3 to 10, and the optimal amount of protein A (from 2.13.1) was added into each tube, then 1% PEG 20,000 (2 drops) was added in order to stabilize gold sols, and followed by coagulation step with 10% NaCl as described previously.

Once the optimal pH for adsorption and optimal amount of protein A in stabilizing gold-protein A complex were determined, a large scale of colloidal gold-protein A complex was prepared accordingly. By using ten-fold excess of protein A dissolved in 100 µl of double-distilled water and filtered through a millipore filter into an ultraclean plastic bottle. One hundred milliliters of colloidal gold was then added and stirred. After 5 min, 5 ml of 1% PEG 20,000 was added . The colloid was centrifuged at 28,000 x g for 1 h. The clear supernatant fraction was discarded and the red pellet was resuspended in 10 ml of 0.02 M Tris-HCl buffer, pH 7.4 containing 0.9% NaCl, and 0.5 mg/ml PEG 20,000. This stabilized prepared complex can be stored at 4°C for a long time until use.

2.14 Tissue preparation inspection by electron microscope

Root and leaf tissues were immersed in 0.1 M Sodium cacodylate buffer, pH 7.4 containing trace amount of Tween-20. After 15 min of shaking, the tissues were rinsed with several changes of cacodylate buffer. Leaves and roots were cut into 1x1 mm sections and fixed in 2.0% paraformaldohyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 0°C, overnight. The samples were washed 3 times with 0.1 M cacodylate buffer. Then samples were postfixed in 2% OsO_4 (Electron

Microscopy Sciences) in 0.1 M cacodylate buffer for 2 h at 0°C. Washing step was carried out as described previously. Subsequently, tissues were dehydrated in ethanol series : 35% (v/v) ethanol, 30 min; 50% (v/v) ethanol, 30 min; 70% (v/v), 30 min; 95% (v/v), 1 h and absolute ethanol 1 h, twice.

Dehydrated sample in absolute ethanol was embedded in Spurr's resin (Spurr, 1969). The embedding medium was freshly prepared before used by gently mixing 10 g vinylcyclohexane dioxide, 6 g diglycidyl) ether of polypropyleneglycol, 26 g nonenyl succinic anhydride and 0.4 g dimethylamino-ethanol. Starting with pre-embedding the sample in a mixture of n-butyl glycidyl ether and absolute ethanol (1:1), for 30 min, and further pre-embedding in n-butyl glycidyl ether, for 30 min, and further pre-embedding in n-butyl glycidyl ether, for 30 min. Afterthat, the samples were embedded in a mixture of n-butylglycidyl ether: Spurr's resin (1:1) for 1 h, followed by embedding in three consecutive change of Spurr's resin, each change consumed 1 h. After the third change of pure Spurr's resin, the samples were transferred to a flat embedding mold containing embedding medium, and left standing for plastic polymerization in an oven at 70°C for at least 8 h.

Ultra-thin sections (the gray to gold reflectance color) were cut on an ultramicrotome (LKB 2088 Ultratome V) equipped with a glass knife. The sections were picked up on Nickel grids.

2.15 Immunogold staining (Hermoso, et al., 1989)

All subsequent procedures were performed at room temperature in a moist chamber.

Sections on grids were rinsed with double distilled water. Non specific binding was blocked by floating grids on a droplet of 1% BSA in 10 mM Tris-HCl, buffer, pH 7.4 containing 0.15 M NaCl, 0.02% sodium

azide, and 0.05% Tween-20 (Tris-BSA), for 1 h. The grids were then transferred to a drop of antilectin diluted 1:10 with TBST-BSA. After incubation for 2 h, sections were washed with TBST for 5 times, (10 min each). Sections were then immersed in colloidal gold-protein A droplet (diluted 1:10 in TBST-BSA) for 1 h, then washed 5 times with TBST, followed by double distilled water and finally blot dry on a filter paper. Immunogold labelled sections were poststained with LKB commercial reagent of uranyl acetate and lead citrate in a stainer (LKB 2168 Ultrastainer, Carlsberg System). Sections were viewed and photographed on a Joel Tokyo, Japan, 200 cx electron microscope operated at 80 kv.

Control sections were performed by substitution of antilectin with TBST or nonimmune serum.