CHAPTER II

MATERIALS AND METHODS

2.1 Bacteria

Klebsiella oxytoca strain R15 is a rhizopheric nitrogen-fixing bacteria isolated from rice cv RD7 in Thailand at Rangsit Rice Experimental Station (Harinasut, 1981).

2.2 Rice

Foundation seeds of *Oryza sativa* CV.RD7 were obtained from the Department of Rice Research, Ministry of Agriculture and Cooperatives, Thailand.

2.3 Media and growth condition

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

Tryptone	10.0 g/l
NaCl	10.0 g/l
Yeast extract	5.0 g/l

Adjust pH to 7.0 with 0.1 N NaOH and 15 g/l agar was added for solid medium.

Nitrogen-free medium (NF), (Dobereiner,1977)

K_2HPO4	0.05 g/l
KH_2PO_4	0.15 g/l
$CaCl_2.2H_2O$	0.01 g/l
$MgSO_4.7H_2O$	0.02 g/l
$NaMoO_4.2H_2O$	0.002g/l
$FeCl_3$	0.01 g/l
Glucose	20.00 g/l

Adjust pH to 7.0 with 0.1 N NaOH

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

Solution A

$ZnSO_4.7H_2O$	0.43 mg/l
$MnSO_4.H_2O$	1.30 mg/l
$Na_2MoO_4.2H_2O$	0.75 mg/l
H_3BO_3	2.80 mg/l
$CuSO_4.7H_2O$	0.026 mg/l
$CaSO_4.7H_2O$	0.07 mg/l

Solution B

$MgSO_4.7H_2O$	100 mg/l
$CaCl_2.2H_2O$	100 mg/l
$FeSO_4.7H_2O$	22 mg/l

Sodium EDTA	20 mg/l
Solution A	20 ml

Solution C

K_2HPO_4	0.9 g/l
KH_2PO_4	0.6 g/l

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

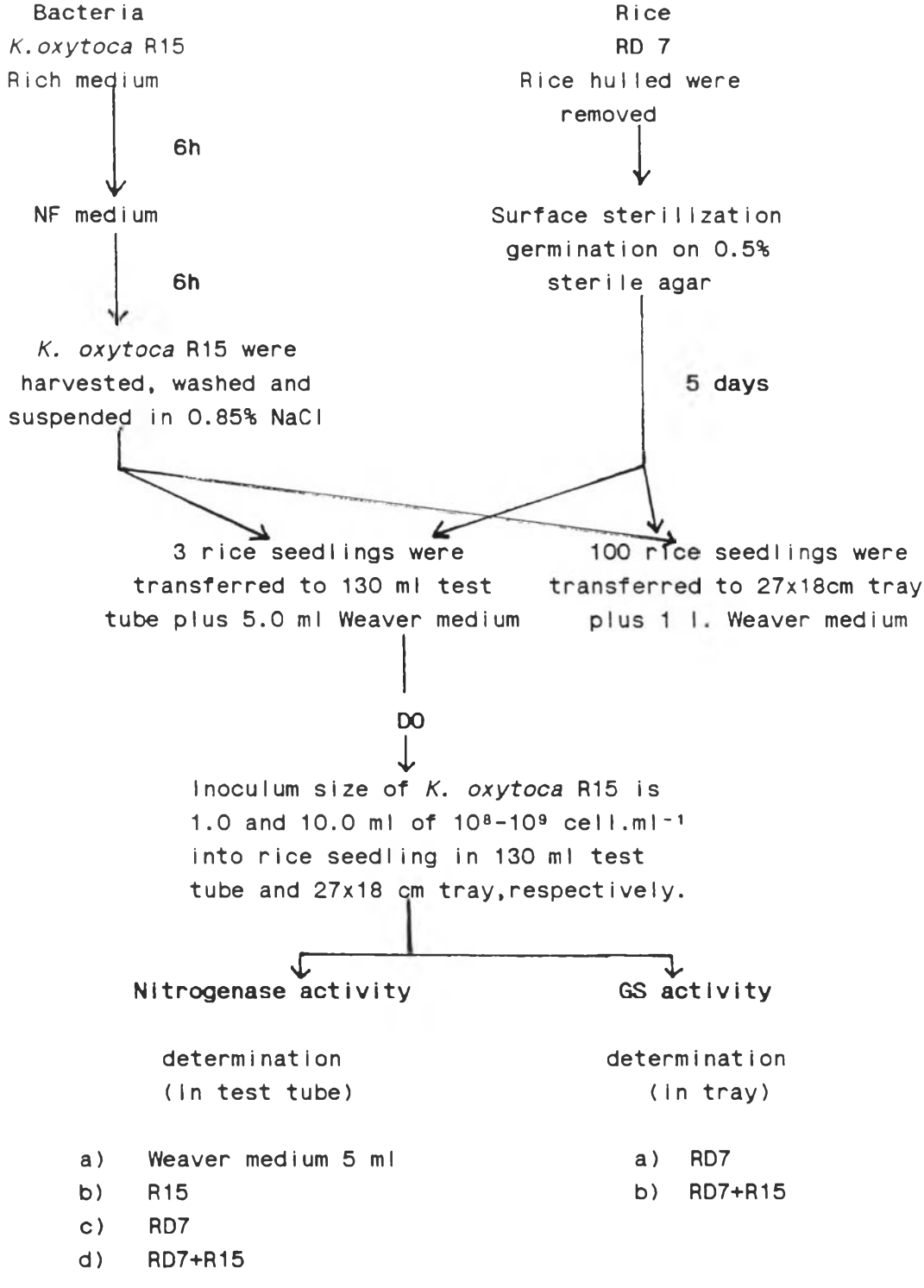
2.4 Maintenance of bacterial culture

For long-term storage, *Klebsiella oxytoca* R15 were maintained by adding equal volume of medium culture in the sterile glycerol and kept in a deep-freezer at -75°C for 1-2 years. Activation of bacteria was performed by growing cell in LB plate for 24 h at 30°C. A single colony of bacteria from LB plate was inoculated into 100 ml of LB broth in a 500 ml Erlenmeyer flask and incubate at 30°C with shaking in and orbital shaker until OD_{420} reached 0.8-1.0, cell were then transferred into a 1 l. Erlenmeyer flask containing 250 ml NF medium. Cell were harvested by centrifugation at 7,000 g 4°C 20 min, washed once with 0.85% NaCl and suspended in 0.85% NaCl before they were inoculated into rice seedlings.

2.5 Bacteria-rice association

Rice seeds were surface sterilized by soaking in 95% ethanol 30 second and immediately washed off with sterile distilled water containing Tween-20, (5-6 drops/100 ml) several times then soaking in 40%, 30%, and 20% Clorox which contained tween 20 (5-6 drops/100 ml) for 25, 20,and 15 min respectively and washed off with excess amount of sterile distilled water. Seeds were germinated on 0.5% agar in petri-dish about 25 seeds per plate and placed in the dark for 4 days.On the fifth day 3 rice seedling which were contaminated-free and approximately equal size were selected and transferred to a 130 ml test tube containing 5.0 ml sterile distilled water,and 100 rice seedlings to a 27x18 cm trays containing 1 l. Weaver medium (Weaver *et.al.*,1975). Tube and trays with rice seedlings were placed in a environment 25/28°C,and under 12/12 light/dark condition, illuminated with provided by plant-growth florescent lamp. The experiment was designed to measure nitrogenase activity from the 130 ml test tube and GS specific activity from 27X18 cm trays, as shown in Figure 2.1

Figure 2.1 Protocol of *Klebsiella oxytoca* R15 inoculation in rice (RD7)



2.6 Tissue preparation for stereomicroscopic studies

The rice plant from the control and inoculated tray was collected and immediately observed under a stereomicroscope,on day 0, 7, 14 and 21 after inoculation

2.7 Tissues preparation for inspection by electron microscope

Root and leaf tissue were immersed in 0.1 M sodium cacodylate buffer pH 7.4 containing trace amount of Tween 20. After 15 min of shaking, the tissue were rinsed with several changes of cacodylate buffer. Leaves and roots were cut into 1x1 mm sections and fixed in 2.0 % paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 0°C, overnight. The sample were washed 3 times with 0.1 M cacodylate buffer. Then sample were post-fixed in 2% OsO_4 (Electron Microscopy Science) in 0.1 M cacodylate buffer for 2 hour at 4°C. Washing steps were carried out as described previously. Subsequently, tissue were dehydrated in ethanol series :35% (v/v), 30 min; 50% (v/v), 30 min; 70% (v/v), 30 min; 95% (v/v) 1 h and absolute ethanol 1 h, twice. Dehydrated sample for SEM was dried by critical point drying method (CPD) and coated with gold. For TEM, sample was embedded in Spurr's resin (Spurr,1969) The embedding medium was freshly prepared before use by gently mixing 10 g vinylcyclohexane

dioxide, 6 g diglyscidyl ether of polypropyleneglycol, 26 g nonenyl succinic anhydride and 0.4 g dimethylamino-ethanol. Starting with pre-embedding the sample in a mixing 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 embedding in n-butyl glycidyl ether, for 30 min. After that, the sample was 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 for 1 h. After the third change of pure Spurr's resin, the sample was 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 pick up on nickel grids.

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

Colloidal gold particles 20 nm in diameter were 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, siliconinzed Erlenmeyer 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.

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.

2.8.1 Optimum 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 ul volume in series of 5 ml tube. 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 indicated the unstabilized particles. The optimum amount of protein A was selected for the further experiment.

2.8.2. Optimum 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 optimum amount of protein A (from 2,14.2) 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 optimum pH for adsorption and optimum amount of protein A in stabilized 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 ul of double distilled water and filtered through a millipore filter into an ultra clean plastic bottle. One hundred ml 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 was discarded and the red pellet was re-suspended 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.

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

All subsequent procedures were performed at room temperature in a moist chamber. Sections on grid 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 anti maize GS (Prof.Sugiyama Tatsuo Department of Agricultural Chemistry Nagoya University). 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 (dilute 1:10 in TBST-BSA) for 1 h, then washed 5 time with TBST, followed by double distilled water and finally blot dry on a filter paper. Immunogold labeled sections were post-stained with LKB commercial reagent of uranyl acetate and lead citrate in a staining (LKB) 2168 Ultrastrainer, Carlsberg System). section were viewed and photographed on a Jeol Tokyo, Japan, 200 cx electron microscope operated at 80 kv. Control sections were performed by substitution of anti GS with TBST or nonimmune serum.

2.10 GS in bacteria-rice association

To determine GS specific activity, 2 trays of rice seedlings were used,(1) rice seedlings only, (b) rice seedlings + *K. oxytoca* R15. Rice seedlings, 100 plants were cut and weighed, of about 1-2 g fresh weight of rice roots or leaves were frozen in

liquid nitrogen for 1 h before grinding in 5 ml of Tris-HCl buffer, pH 7.6 . The roots and leaves homogenate were filtered through a lawn sieve and washed with 100 mM Tris-HCl buffer, pH 7.6, 2-3 times. The homogenate was then centrifuge at 60,000 g 30 min at 4°C.

2.11 Determination of GS activity by transferase assay

The transferase activity of GS was assayed essentially according to Farnden and Robertson (1980) in which the reaction mixture was freshly prepared before use, by mixing 1.0 ml 0.5 mM imidazole-HCl buffer, pH 7.0, 2.0 ml 0.15 M glutamine, 0.3 ml 0.1 M $MnSO_4$, 0.4 ml 0.01 M ADP, 0.2 ml 1.0 M sodium arsenate pH 7.0 and 1.1 ml distilled water to make 5.0 ml of mixture. For standard curve 125 µl of increasing concentration of γ-glutamyl hydroxamate (0.1-1.0 µM) were prepared, for unknown sample, 125 µl of the reaction(4), mixture containing the above substance was mixed with 100 µl plant extract test solution to give a total volume of 225 µl and pre-incubated at room temperature (25°C) for 5 min. The reaction was started by adding 25 µl hydroxylamine solution (prepared by mixing 1.0 M NH_2OH-HCl : 1.0 M NaOH = 1 : 1 just before used). After incubation for 15 min at room temperature the reaction was stopped by adding 0.5 ml $FeCl_3$ regent (prepared by

mixing 10 % $FeCl_3.6H_2O$: 24% TCA : 6 M HCl : distilled water = 8: 2 : 1 : 13). Control assay as performed by omitting ADP, and sodium arsenate. Assay mixture was centrifuged at 5,000 X g for 20 min and the absorbance (A_{540}) of glutamyl hydroxamate formed was determined in Spectronic 2000 spectrophotometer. Standard curve of glutamyl hydroxamate (0.1-1.0 μM) was prepared in every assay (Appendix I). The specific activity of GS transferase activity was expressed as (μM γ glutamyl hydroxamate formed).(mg protein.min.)$^{-1}$. Protein was determined by the method of Bradford (1976) using BSA in the range of 0-10 μg/100 μl as standard (Appendix II)

2.12 Polyacrylamide gel electrophoresis

Non denaturing polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% polyacrylamide gel (Davis,1964) of 0.5 mm thick stab gel (10.2x8.2cm) with Tris-glycine buffer pH 8.3. The electrophoresis was run from cathode towards the anode with constant current of 15 mA per slab gel at 4°C for 1-2 h,in a midget Electrophoresis apparatus(LKB) attached to a thermostat water bath of LKB 2209 Multitemp. The protein bands wrere strained with Coomassie brilliant blue R 250. The GS activity was stained by incubating the gel in the transferase assay mixture for 20 min

at room temperature and color was developed by dipping gel in the $FeCl^3$ in reagent as described in method 2.11. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE), was carried out on 10% polyacrylamide gel according to Laemmli (1970). The electrophoresis was performed as described previously under control temperature of 15°C.

2.13 Western blot analysis of GS protein by GS antibody

After SDS-PAGE has been done, protein bands on stab gel were transferred to a nitrocellulose membrane (LKB) as described by Lin and Kasmatsu(1989). Transfer buffer used was 25 mM Tris-HCl and 150 mM glycine buffer pH 8.3. The electroblotting was performed at 0.6-1.2 mA, 4°C for 1 h. Then transferred membrane was washed briefly with distilled water and 50 mM Tris-HCl buffer saline pH 7.5, TBS and non specific binding was blocked by incubating the membrane overnight in TBS-BSA (TBS,1%BSA) with gentle shaking at room temperature.

2.13.1 Antibody-antigen reaction on the membrane filter

Transfer the nitrocellulose membrane to a plastic tray containing 30 ml fresh TBS-BSA and add 30 µl antibody against GS protein after incubation with gentle shaking at room temperature for 1 h, then the membrane was washed three times with 40 ml

TBS-Tween 20 each for 5-15 min. After that, 30 ml of fresh TBS-Tween 20 containing 10 µl of the second antibody (horseradishperoxidase-goat anti-rabbit IgG; dilution 13,000) was added to the membrane, and incubate for 1 h with gentle shaking. Decant away the second antibody solution and wash as described previously.

2.13.2 Detection of antibody-binding polypeptide

Transfer the washed membrane to a clean tray containing 10 ml 0.03 % 3,3 -diaminobenzidine tetrahydrochloride (DAB) in TBS and add 10 ul of 30% H_2O_2 freshly prepared according to Walk, 1984. Wait for about 10-30 min then stop reaction by changing the solution to distilled water when the brown color of protein bands reached the desired intensity.

2.14 Determination of nitrogenase activity

Nitrogenase activity was determined by acetylene reduction assay (ARA) according to Elkan (1987), using 4 set of 10 replication tubes, each set contains: (a) 5 ml of Weaver medium + 1 ml of NaCl (b) 3 rice seedling (c) 3 rice seedling + *K. oxytoca* R15 10^8 cell.ml^{-1} (d). *K. oxytoca* R15 10^8 cell.ml^{-1}. Each tube was closed with a serum stopper, the head space was changed to 10%

acetylene by removing air and replacing with equal volume of acetylene (Ratanachoke, Engineering Ltd.). At various time intervals, 300 µl of gaseous phase was injected in a gas chromatograph (Packard Model 437 A)under the following condition.

column packing	parapak N
column temperature	90°C
injector temperature	110°C
detector temperature	150°C
flow rate of N_2	30 ml.min^{-1}
flow rate of H_2	25 ml.min^{-1}
flow rate of air	250 ml.min^{-1}

The assay was performed every other day after inoculation of *K. oxytoca* R15 for 6-8 days or until the nitrogenase activity was plateau. The standard pure ethylene gas (99%) (Thai Industrial Gas Ltd.) was diluted by air and injected as standard before every assay.