CHAPTER 2

Materials and Methods

2.1. Bacteria

Nitrogen-fixing bacterial cultures used as references are as follows: Klebsiella oxytoca NG13, Pseudomonas sp.H8, P KLH 76, Azospirillum lipoferum 34H and A.FS. The first strain was isolated from rice grown in Japan and provided by Dr.Y.Hirota,National Institute Genetics ,Japan (NIG) and the other 4 strains were isolated from rice grown at the International Rice Research Institute (IRRI) Philippines and presented to us by Dr.I.Watanabe. Nitrogen-fixing Klebsiella pneumoniae M5a1 and Escherichia coli C600 ; were kindly provided from Dr. C. Kenedy and maintained at the Unit Cell of Genetics Engineering, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand.

The indigenous N_2-fixing Klebsiella like strains; R15,R17,and Azospirillum like strain; R25 were isolated from the rhizosphere of rice grown at Tapra,

North-Eastern Agricultural Bureau and Rangsit Rice Experimental station, Thailand, by P.Harinasut and J. Boonjawat, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand (Harinasut,1981).

2.2 Rice

Foundation seeds of rice cultivar; RD5, RD6, RD7, RD23, RD25, Khaodokmali 105 (KDML), Luang-pratew 123 (LPT), and San-Pa-tong (SPT), were obtained from Bangkhen Rice Experimental Station, Department of Agriculture, Ministry of Agriculture and Co-operative, Thailand.

2.3 Rabbit

New Zealand White rabbits were obtained from the Animal Center, faculty of Animal Science, Kasetsart University,Thailand. Only female rabbits about six month old and have never passed immunization were used. The rabbits were quartered and maintained at the BNF Research Center, Soil Science Devision, Department of Agriculture, Ministry of Agriculture and Co-operatives,

Thailand.

2.4 Chemicals and Biochemicals

All chemicals were obtained commercially from BDH, E. Merck or Sigma and were of reagent grade or better.

2.5 Preparation of Culture media and cultivation

2.5.1. Rich medium (RM), (Luria et al.,1960)

tryptone	10 g/l
yeast extract	5 g/l
sodium chloride	10 g/l
glucose	2 g/l

All ingredients were dissolved in distilled water, the medium was adjusted to pH 7.0 with 1N NaOH, before make up to 1 liter, and autoclaved at 121 °C for 15 min. For a solid medium, 15 g of Noble agar was added into 1 liter of the liquid medium.

2.5.2.Tryptic soy agar (TSA), (Barraquio et al., 1983)

Dissolved 1 g of tryptic soy and 15 g of Noble agar in distilled water. The media was adjusted to pH 7.0 with 1N NaOH and made up to 1 liter, autoclaved at 121°C for 15 min.

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

Dipotassium hydrogen phosphate, K_2HPO_4	0.05	g/l
Potassium dihydrogen phosphate, KH_2PO_4	0.15	g/l
Calcium chloride, $CaCl_2.2H_2O$	0.01	g/l
Magnesium sulphate, $MgSO_4.7H_2O$	0.002	g/l
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.002	g/l
Ferric chloride, $FeCl_3$	0.01	g/l
Sodium bicarbonate, $NaHCO_3$	0.01	g/l
Glucose	20.0	g/l

Each ingredient was dissloved in distilled water, and autoclaved at 121°C separately for 15 min. These solutions were mixed in aseptic condition before used. For a solid medium, 15 g of Bacto - agar was added into 1 liter of the liquid medium.

2.5.4 Nitrogen free medium (NF),for R25 or Azospirillum spp. Pseudomonas spp. (Ladha et al., 1982)

Malic acid	5	g/l
Dipotassium hydrogen phosphate, K_2HPO_4	0.5	g/l
Calcium chloride, $CaCl_2.2H_2O$	0.02	g/l
Magnesium sulphate, $MgSO_4.7H_2O$	0.02	g/l
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.002	g/l
Ferrous sulphate, $FeSO_4 .7H_2O$	0.4	g/l
Sodium chloride, NaCl	0.1	g/l
Manganese sulphate, $MnSO_4.H_2O$	0.01	g/l
Biotin	0.0001	g/l
Pyridoxal-HCl	0.0002	g/l

Each ingredient was dissloved in distilled water,and autoclaved at 121°C separately for 15 min. These solution were mixed in aseptic condition before used. For a solid medium, 15 g of Bacto - agar was added into 1 liter of the liquid medium.

2.5.5 Modified Weaver medium (Weaver et al., 1975)

Solution A

Zinc sulphate, $ZnSO_4.7H_2O$	0.43	mg/l

Manganese sulphate, $MnSO_4.H_2O$	1.3 mg/l
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.75 mg/l
Boric acid, H_3BO_3	2.8 mg/l
Copper sulphate, $CuSO_4.7H_2O$	0.026mg/l
Cobalt sulphate, $CoSO_4.7H_2O$	0.07 mg/l

Solution B

Magnesium sulphate, $MgSO_4.7H_2O$	100 mg/l
Calcium chloride, $CaCl_2.2H_2O$	100 mg/l
Ferrous sulphate, $FeSO_4.7H_2O$	22 mg/l
Sodium EDTA (Na_4^+)	20 mg/l
Solution A	20 ml/l

Solution C

Dipotassium hydrogen phosphate, K_2HPO_4	0.9 g/l
Potassium dihydrogen phosphate, KH_2PO_4	0.6 g/l

Working medium composition per liter contained 50 ml of solution B, 15 ml of solution C and 5 g of Noble agar. The medium was autoclaved at 121 °C for 15 min.

2.6 The maintenance of cultures

2.6.1 Short - term maintenance

All *Klebsiella spp.* and *E. Coli* were maintained on RM agar medium. *Pseudomonas* and *Azospirillum* strains were maintained on tryptic soy agar respectively. Stock cultures were stored at 4°C for at least 4 weeks without observable loss of viability. Before use, a single colony was checked for impurity by streaking on the new agar plates, and the single colony isolate was used.

2.6.2 Long - term maintenance

In case of long term storage, the bacterial culture was mixed with an equal volume of autoclaved glycerol and put in a deep - freeze at -20°C for years.

2.7 Cultivation of bacteria

A single colony from RM or typtic soy agar medium was inoculated in 5 ml liquid RM medium at room temperature (30°C) in a rotary shaker until the optical density (OD420nm) reached 0.7. The starter inoculum (3%) was then transferred into 150 ml NF medium supplemented with 10% RM medium. The second starter was incubated in

a reciprocal shaker at 30°C (except Azospirillum spp.). Cells were harvested at optical density (OD420nm) of 0.7 and trasferred into NF medium at 3% inoculum. The growth pattern at 30°C was followed by measuring turbidity at 420nm in a Spectronic 20 (Bausch and Lomb Inc. Ltd., U.S.A.). Colony forming units were determined in a colony counter model 3326 (American Optical Coperation, U.S.A.) by dilution plating method (Somasegaran and Hoben, 1985).

2.8 Preparation of antigens

Bacterial antigen (R15, R17 and R25) were grown in NF - broth until mid - log phase (OD420nm ca. 0.7). Cells were collected, under aseptic conditions, by centrifugation at 3,000 x g for 10 min and resuspened in sterilized normal saline (0.85% NaCl). Centrifuged cells were washed and adjusted with sterile normal saline to $1x10^{9}$ cells/ml. The suspension was heated in boiling water for 30 min to inactivate flagellar and other protein antigens. Merthiolate was added (final concentration of 1:10,000 g/ml) for preservation and stored at 4 - 10 °C in the refrigerator until use.

2.9 Antisera production

2.9.1 Immunization

Rabbits were immunized with R15 , R17 or R25 (1x10^{9} cells/ml normal saline) emulsified with an equal volume of Freund's complete adjuvant. And 0.5 ml portions of the emulsified cells were administered intramuscularly into each hind leg of a New Zealand White rabbit (4 - 5 months).

Boostered injection was performed 4 weeks later and subsequently each week by intravenous injection into an ear vein with 1x10^{9} cells suspended in 1.0 ml of sterile normal saline.

About 2ml of blood was taken from an ear vein before immunization (as control or preimmune serum), 4 weeks after first immunization and 1 week after each boostered injection. Massive bleeding was performed when high titer (not less than 1:1,600) was obtained. Blood was left at room temperature (30°C) for about 2 h and kept in the refrigerator overnight. Serum was separated by centrifugation at 5,000xg, 4°C for 15 min and Merthiolate (Sodium ethylmercurithiosalicylate) was

added as a preservative to the final concentration of 1:10,000 and stored at -20°C until use.

2.9.2 Titration of antiserum by agglutination method

An aliquot of antiserum was serially diluted with normal saline (from 1:25 to 1:6,400) and 100 µl of freshly diluted antiserum were added in duplicate to glass tubes (3x50 mm). Then 100 µl of bacterial antigen ($1x10^9$ cells/ml normal saline) was added to each tube and mixed. Control tubes were incubated by mixing 100 µl of bacterial antigen with 100 µl of normal saline or 100 µl of antiserum (1:25 dilution) with 100 µl of normal saline. After incubation in a waterbath at 52°C for 3 h, agglutination was observed against a background in front of a good light source. The titer was expressed as the reciprocal or the highest dilution of serum which showed distingishable agglutination.

2.10 Identification of N_2-fixing bacteria by immunofluorescence

The 100 µl of tested antigen concentration of 10^7 , 10^8 , 10^9 cells/ml were smeared on the glass slide

which were air dried, heat fixed and covered the smear with a few drops of the serial dilution of anti-R15 serum (and other specific antiserum) starting from a final dilution of 1:25 to 1:200 test for the suitable concentration. Each smear was then covered with a 1:10 dilution of fluorescein isothiocyanate (FITC) - conjugate anti-rabbit immunoglobulin and allowed staining to proceed for 20 min at room temperature in a moist chamber (a plastic box containing moist pieces of paper towel) and kept in the dark. The excess FITC conjugate was removed by washing with phosphate-buffer saline (PBS, containing NaCl 8.0 g ; KH_2PO_4 0.2 g ;$Na_2HPO_4.12H_2O$ 2.9 g ; KCl 0.2 g ; and NaN_3 0.2 g per liter , pH 7.4) for 15 min twice. After rinsing with PBS solution for a minute. The slide was dipped in normal saline for 5 min, air dried and mounted in glycerol. The slide was observed under an epifluorescent microscope (Nikon,Optiphot) equipped with a mercury vapour light source, and a B filter cassette (excitation filter wavelength 420-485 nm and absorption filter of 520-580 nm). The photographs were taken with a Nikon FX-35A camera attached, using KODAK Extrachome 400 ASA for diapositives. The intensity of fluorescence observed with the highest dilution of each homologous antigen and

non-homologous antigen was ranked by the following scale;

++++ : Brilliant yellow - green is homologous strains of antigen or very closly related serology.

+++ : Bright yellow - green is non homologous strains with closely related serology.

++ : Yellow - green is non homologous strains with moderately related serology.

+ : Dull - green is non homologous strains with sparsely related serology.

0 : No fluorescence is background

The highest dilution which could be rated as 4+ was used for strains identification. The non-homologous strains can be shown with rating of fluorescene intensity from 4+ to 1+.

2.11 Salt effect on strain identification by indirect immunofluorescence

The starter of bacterial culture from 2.7 was used as inoculum into triplicate flask of 150 ml RM medium containing NaCl to the final concentration of

2.5%, 5.0%, 7.0%, 10.0%, 12.0% and 15.0% and NF medium containing with NaCl 0.5%, 2.5%, 5.0%, 7.5%, and 10.0% concentration. Growth was observed by measuring turbidity at 420nm (OD420) in a Spectronic 20 and colony forming unit was also performed by dilution plating method in accordance with the measurment of the cell density. When the cell density of each culture reached approximately 0.7, a flask of culture were centrifuged and washed with normal saline. The concentrated cells, approximately 1.0 unit of OD420, were used to react with homologous antiserum to detect IF reaction.

2.12 Preparation of rice seedling roots

The healthy - look seeds of rice cultivars ; RD5, RD6, RD7, RD23, RD25, KDML, LPT and SPT were selected. Newly harvested seeds were incubated at 50°C in a vacuum - oven for 4-5 days to break rice dormancy. Surface sterization of rice seeds were done by soaking in 95% ethanol for 1 min, immediately washed with tap water containing Tween20 (5-6 drops of Tween20 to 100ml of tap water) several times, stirred in 30% Chlorox for 25 min, 10% Chlorox 15 min and 5% Chlorox for 15-60 min under suction with an air pump. The seeds were then

washed in sterile distilled water several times in order to remove the sterilizing agent. Seeds were germinated in a moist - sterile petri-dish for two days at room temperature. After, the geminated and ungerminated seeds were counted and calculated in the term of percentage of germination, transferred 3 germinated seedlings to each 30x200 mm glass tube. Each tube contained 30 ml of modified Weaver Burk medium containing 0.5% Noble agar for 7 days. Only excised roots tip 1 cm long were used in the adsorption assay.

2.13 Assay for adsorption of bacteria on rice roots by indirect immunofluorecence (Haahtela et al., 1986)

Bacterial suspension $1x10^7$, $1x10^8$, and $1x10^9$ cells/ in one ml of PBS were incubated with the three roots from 2.12 at room temperature. The roots were then washed twice for 15 min with 10 ml of PBS. The roots were crushed by glass rod, then smeared on a slide, air dried and heat fixed. The roots debris were covered with one drop of homologous antiserum (diluted 1:20 in PBS - bovine serum albumin for 15 min. The roots were washed twice with PBS and covered with one drop of Rhodamine gel to block autofluorescence of plant tissue

(Bohlool and Schmidt, 1968). Before Rhodamine gel dried, one drop of fluorescein isothiocyanate (FITC) - conjugate anti - rabbit immunoglobulin (dilution 1:10) which contained 1% (wt/vol) bovine serum albumin was applied and incubated at room temperature (ca. 30°C) for 20 min. The excess FITC was removed by washing with PBS as described in 2.10 then mounted, and observed in an epi - fluorescent microscope.

2.14 Assay for associative bacteria on rice roots by indirect immunofluorecence

Bacterial suspension $5x10^8$ cells/ml were inoculated into the tube prepared as in 2.12 at day zero. After 7 days,the roots were cut off and washed twice for 15 min with 10 ml of PBS. The root sample were fixed and determined for the bacterial antigens as described in 2.13.

2.15 Development of enzyme linked immunosorbent assay (ELISA) procedure (Fuhrmann and Wollum II, 1985)

There are 4 steps in ELISA which starts from (i)

antigen coating (ii) addition of the first antibody from rabbit, (iii) addition of goat anti - rabbit IgG - alkaline phosphatase conjugate, and (iv) color development by adding substrate p-nitrophenyl phosphate.

Antigen coating was performed by using a bacterial antigens, suspended in coating buffer (composition per liter according to Voller et al., 1980 was as follow: Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; and NaN_3 0.2 g, final acidity was adjusted to pH 9.6 with 0.5 M HCl) and made a series of two fold dilution ranging from $1x10^9$ - $1x10^6$ cells/ml into the well of a flat well ELISA microtiterplates (Nunc, Denmark). An aliquot of 100µl was delivered to each well and incubated at 5°C overnight for complete coating. The excess bacterial antigens were removed from the well by shaking the plates and thoroughly washed twice with PBS - Tween (PBS containing 0.5% Tween 20). During the first washing, the plate was flooded with PBS - Tween which was then immediately removed to minimize cross - contamination among wells, and for the two final washing, PBS - Tween was remained in the wells for 5 min before removal.

To determine the proper working dilution of the first antiserum, a series of two fold dilution (1:6,400 to 1:102,400) in PBS - Tween was performed and added (100µl) to the series of wells precoated with the serial two fold dilution of the homologous antigen (started at $1x10^{9}$ cells/ml). In other words, the antiserum dilution was varied on the Y-axis and antigen dilution was varied on the X-axis of the microtiterplate. The antigen - antiserum mixture was incubated in a chamber with saturated humidity at 30°C for various incubation time (0.5 - 3.0 h).

The excess antiserum was removed by washing twice with PBS - Tween which was then immediately removed to minimize cross-contamination among wells, and for the two final washings, PBS - Tween was remained in the wells for 5 min before removal. Then 100µl of diluted goat antirabbit IgG-alkaline phosphatase conjugate (Sigma) in PBS - Tween was added to each well. The dilution of the second antibody-conjugate was varied from 1:2,500 to 1:1,500. The dilution of the first antiserum was fixed, while the dilution of the second antibody-conjugate was varied. The microtiter plate was incubated at 37°C. At different period of

incubation time (0.5, 1.0, 2.0, 3.0, and 12 h) the color reaction was proceeded.

The substrate solution containing 1 mg of p-nitrophenyl phosphate per ml of substrate buffer (composing of 97 ml of diethanolamine, 0.2 g NaN_3 and 0.1 g $MgCl_2.6H_2O$ per liter, adjusted to pH 9.8 with 0.1 M NaOH) was added to each well (100 µl) and the plate was allowed to develop the yellow color at room temperature. The reaction time was varied between 40 min and 50 min, and stopped with 50 µl of 3M NaOH per well. The absorbance of color product was measured at wavelength 405 nm in a Titertek Multiskan Plus plate reader (Flow Laboratories). A test well was judged positive generally when absorbance ranging from 1.0 - 1.5 unit was observed, and negative when its absorbance value was at least half of the mean of the corresponding positive control well of the same plate.

2.16 Competitive binding assay of indirect enzyme linked immunosorbent assay (COM-IND-ELISA)

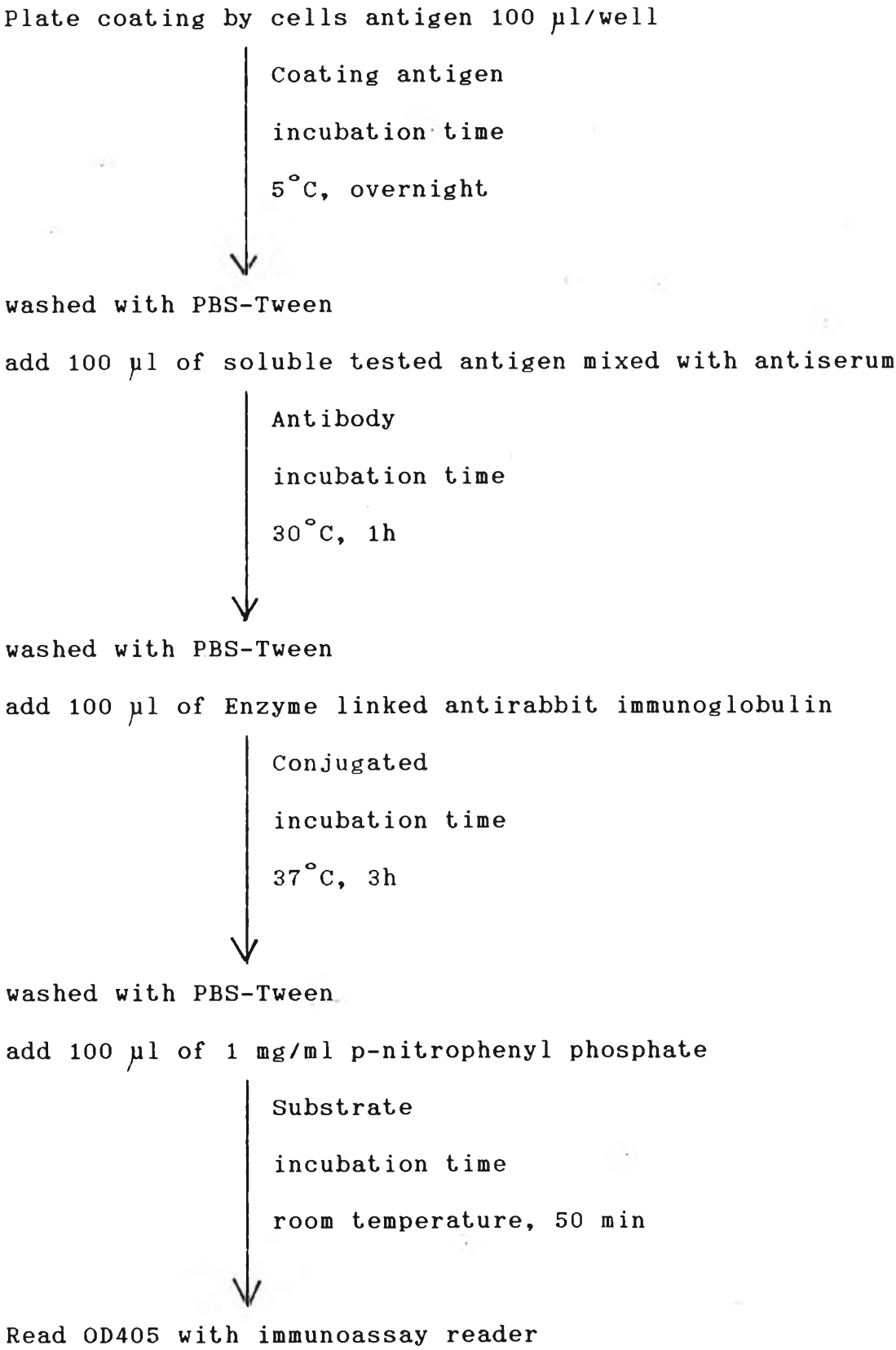
The IND-ELISA technique is by far less sensitive than COM-IND-ELISA, which has the profound advantage in

reducing the coating antigen factor. In this study, each plastic well of an ELISA plate was coated with an exact concentration of antigen to give an OD405 value ~ 1.0. After the coating step, the appropriate antiserum concentration of each bacterial culture was mixed with a series of two fold dilution of bacteria to the final dilution ranging from $1x10^9$ - $1x10^6$ cells/ml with PBS - Tween. Then, the mixture was added to the test well as the first antiserum and incubated at 30°C for 1 h. The protocol of COM-IND-ELISA was performed according to as described in 2.15 and also summarized in Fig. 2.1.

2.17 Examination for the specificity of antisera against strain R15, R17, and R25

The specificity of antisera and COM-IND-ELISA for detecting culture antigen of R15, R17, and R25 were investigated by coating the exact concentration of these bacteria as described in 2.16. The first antiserum was incubated with $2.5x10^8$ cells/ml of the nonhomologous or related strain such as A.FS, A.34H, P.Sp7, A.SpRG20, A. SpBr17, A.ama, K.oxytoca NG13, K.pneumonia, P.H8, P. KLH76 and E. coli at 30°C for 30 min. The ELISA values were detected and changed in terms of percentage of

Fig. 2.1 Protocol of COM-IND-ELISA



competitive binding and cross - reaction as shown in Table 2.1.

Table 2.1 Example for calculation of per cent competitive binding and per cent cross-reaction

Soluble antigen	ELISA value	per cent binding	per cent cross-reaction
No addition (control)	1.0	100	-
Homologous antigen	0.325	67.5	100
Related strain	0.87	13.0	19.25

per cent competitive binding:

$$\frac{\text{ELISA value of (control - the tested antigen)}}{\text{ELISA value of control}} \times 100$$

ELISA values of control = no addition any soluble antigen in the tested wells.

ELISA values of homologous antigen = ELISA values of 2.5×10^8 cells/ml of R15, R17, and R25.

ELISA values of related strains = ELISA values of

$2.5x10^8$ cells/ml of the related strains.

per cent cross reaction

$$\frac{\text{percent competitive binding of related strain}}{\text{percent competitive binding of homologous antigen}} \times 100$$

2.18 Salt effect on quantitative determination by COM-IND-ELISA

Bacterial cells grown in different salt concentrations as described in 2.11 were collected after measuring OD420 and, colony forming unit. Washed cells were suspended in normal saline and heated in boiling water for 30 min, washed and resuspended to the original volume with PBS-Tween. Before quantitative determination by COM-IND-ELISA as described in 2.15.

2.19 Acetylene Reduction Activity (ARA) Assay

Nitrogen-fixing activity of rice plant in association with bacteria was assayed in a 30x200 mm tube, containing 3 rice seedlings and 30 ml modified Weaver medium made semi solid with 0.5% Noble agar, and inoculated with bacterial (2-day-old of $5x10^8$ cells/ml).

Normal saline was added instead of bacteria for control tubes. After 7 days, the cotton plug was replaced with sterilized rubber stopper fitted with a Suba Seal serum stopper. Twelve replicate tubes were performed for each set of experimental condition. For each tube, 10% gas-phase volume was replaced with acetylene. At zero time, the base line concentration of C_2H_2 and C_2H_4 in the head space volume was dertermined by drawing 100µl aliquot sampling with a sterile disposable syringe and injected to a Varian 3700 gas chromatograph, equipped with a Porapak N column (90°C)and flame - ionization detector (FID) using 30 ml.min^{-1} of oxygen - free nitrogen as carrier gas. Ethylene production in each tube was followed during 0 - 14 days and calculated in µmole.g root dry weight^{-1} by comparing the peak height with known amount of standard C_2H_4 at the same retention time.

2.20 Determination of plant vigor index

(Pururshothaman, 1987)

After each ARA assay, the rice plants were taken out of the tube to measure the lenght of shoots and roots (cm). The plant vigor index is the lenght of

shoot in cm plus root lenght in cm and multiply by % germination as shown in Table 2.2. Difference in vigor index of rice plants with bacterial inoculation minus the control without bacterial inoculation is the associative net vigor index.

Table 2.2 Example for calculation of plant vigor index

condition of rice	shoot lenght (cm)	root lenght (cm)	%germination	Vigor index (cm.%)
inoculated	19.2	6.6	97	2,502
control	12.9	6.7	97	1,901
	associative net vigor index			601

2.21 Application of COM-IND-ELISA for quantitation of associative N_2 - fixing bacteria on the rice root

After Vigor index measurement, the rice plants were washed in PBS-Tween to remove the medium then

chopped into small pieces and resuspended in 3 ml of PBS-Tween. The roots were heated in a boiling water bath for 30 min and washed twice with PBS - Tween for 15 min. The roots were transferred into a siliconized glass tube and grinded with a glass rod and transferred to 1.5 ml microfuge tube containing a plug of siliconized glass rod which served to trap the root debris and a hole was made at the bottom of the plastic microfuge tube. This tube were then placed in the mounth of a glass tube (13x100 mm) as shown in Fig. 2.2. After centrifugation for 2 min at maximun speed of a top bench centrifuge, PBS - Tween was added for the second wash and recentrifuged. The eluant was concentrated to the final volume 1 ml and the roots were then dried at 60°C for 24 h and weighed.

For quantitative determination of associative bacteria in rice roots, the standard curves were done by usingthe series of two fold dilution of heated cells ranging from $1x10^9$ - $1x10^6$ cells/ml that were filtrated through the microtube system as described above. Then, the eluant was concentrated to the started volume of 1ml and proceed as described in 2.16.

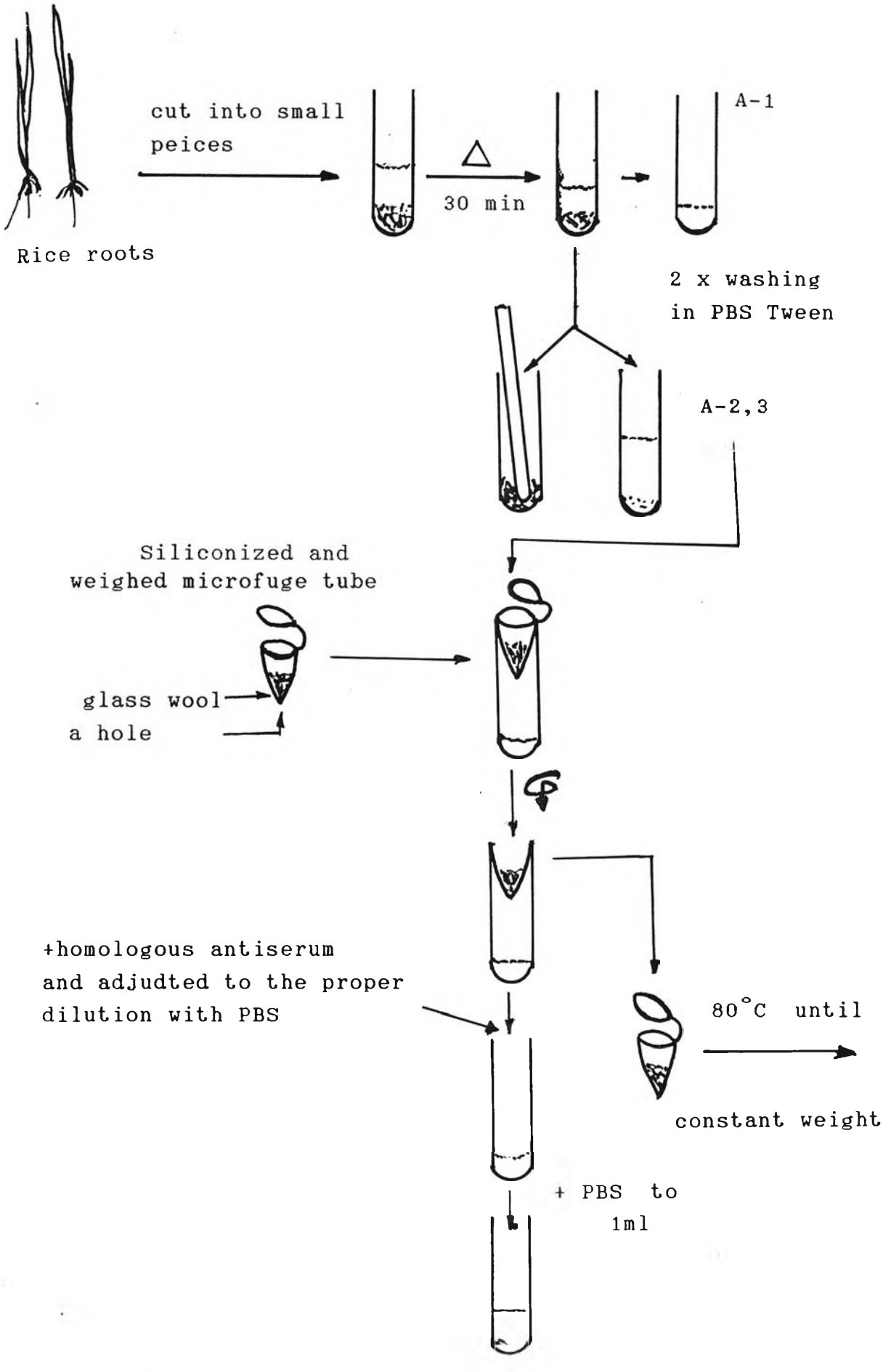


Figure 2.2 Protocol for detection of associative bacteria