CHAPTER II.

MATERIALS AND METHODS

MATERIALS.

1. Rice root and rhizospheric soil samples.

Rice root and rhixospheric soil samples were collected from three sites;

1. Tapra, breeding plots North-Eastern Agricultural Bureau. Tapra District, Khonkan Province, (the distance is about 440 km. far from Bangkok.)

2. Long term fertility plots, Chumpae Rice Experiment Station. Chumpae District, Khonkan Province. (the distance is about 521 km. far from Bangkok.)

3. Long term fertility plots, Rangsit Rice Experiment Station. Thanyaburi District, Pathumthani Province. (tee distance is about 43 km. far from Bangkok.)

Soil types and composition at each site is as follow.

Table 2. Soil types and composition of the experimental sites. (39)

Experimental Site	Soil type	Soil composition (%)			pH average
		Sand	Silt	Clay	
Tapra	Loamy fine sand	85	11	4	6.1
Chumpae	Low humic glay	57	22	21	5.8
Rangsit	Brackish water alluvial	5	25	70	4.0

At various stages of rice growth at Tapra breeding plots nitrogen (N), phosphorus (P) and potassium (K) fertilzers were applied as shown in Table 3.

Table 3. The application of chemical fertilizer at Tapra, North-Eastern Agricultural Bureau.

Stage of rice growth	N (kg/ha)	P kg/ha)	K (kg/ha)
Seedling	10	10	5
Top dressing	38	-	-
Transplanting	13	17	-
Panicle initiation	75	-	-
Total	136	27	5

As for the long-term fertility polts at Chumpae, rice root and soil samples were collected from Treatment number 1,2 and 5 which were fertilized before transplanting as shown in Table 4

Table 4. The application of chemical fertilizer at Chumpae Rice Experiment Station.

Treatment	N (kg/ha)	P (kg/ha)	K (kg/ha)
1	0	0	0
2	0	38	25
5	113	38	25

At Rangsit Rice Experiment Station, both chemical fertilizer and rice straw compost were applied. Rice root and rhizospheric soil samples were collected from Treatment number 1,5,6, and 10 which were fertilized as shown in Table 5.

Table 5. The application of chemical fertilizer before transplanting and rice straw compost at Rangsit Rice Experiment Station.

Treatment	N(kg/ha)	P(kg/ha)	K(kg/ha)	rice straw compost (ton/ha)
1	0	0	0	0
5	0	0	0	12
6	50	25	25	0
10	50	25	25	12

Different varieties of rice (Oryza sativa) were cultivated at the three experimental sites as summerized in Table 6.

Table 6. The rice varieties grown at the three experimental sttes.

Rice Experiment Station	Rice variety	Type		Genetic status	
		nonsticky rice	sticky rice	modern variety	old popular variety.
Tapra	RD. 15	+		+	
	Kowmali	+			+
	RD. 6		+	+	
	Sanpatong		+		+
Chumpae	RD. 7	+		+	
Rangsit	RD. 7	+		+	

Seeds of Oryza sativa were planted in the wet season of 1979. The 31, 33, and 43 days-old seedling at Tapra, Chumpae and Rangsit respectively, were transplanted in to the flooded plots. At Chumpae and Rangsit each treatment was triplicated. During growing season the rice hills (3-10 hills) were collected randomly from each treatment, put in the plastic bag, and kept in ice box during transport and stored at 4-10° C in the cold room of the Biochemistry Department, Chulalongkorn University until assay (within 1-3 days).

Table 7. Date of sample collections (1979)

Time of Collection	Tapra	Chumpae	Rangsit
1	8 June	15 Sep.	11 Sep.
2	24 June	15 Oct.	11 Oct.
3	20 July	26 Nov.	11 Nov.
4	3 Aug.		
5	17 Aug.		
6	6 Sep.		
7	28 Sep.		
8	2 Nov.		
9	27 Nov.		

2. Chemicals

2.1 For acetylene reduction activity (ARA) measurement.

2.1.1 Gases.

Compressed air	From the Military Science Department.
Hydrogen (H_2)	From the Military Science Department.
Ethylene standard (C_2H_4)	From Scott Environment Technology, inc. USA.
Nitrogen (N_2 OFN)	From Thai Industrial Gases Limited.
Acetylene (C_2H_2)	From Sithichoke Engineering Limited.

2.1.2 Packing material.

Porapak N (mesh 80-100)	From Water Associates, inc. USA.

2.2 Bacterial media.

2.2.1 Nitrogen-free medium(NF medium)((40)

		Grade	Company.
Dipotassium hydrogen phosphate, K_2HPO_4	(0.05g/l)	Laboratory	M&B
Potassium dihydrogen phosphate, KH_2PO_4	(0.15g/l)	Laboratory	M&B
Calcium chloride, $CaCl_2$	(0.01g/l)	Analar	BDH
Magnesium sulphate, $MgSO_4.7H_2O$	(0.2g/l)	Laboratory	BDH
Sodium molybdate, $Na_2MoO_4.2H_2O$	(0.002g/l)	Laboratory	BDH
Ferric chloride, $FeCl_3$	(0.01g/l)	Analar	BDH
Sodium bicarbonate, $NaHCO_3$	(0.01g/l)	Laboratory	M&B
Glucose	(20g/l)	Analar	Fluka

2.2.2 Rich medium (41)

Tryptone	(10g/l)	Difco

Yeast extract	(5g/l)	BBL
Sodium chloride, NaCl	(10g/l)	Analar Mallinckrodt
Glucose	(0.2%)	Analar Fluka

2.3 Gram's stain (Hucker's modification) was prepared in the laboratory.

2.3.1 Ammonium Oxalate Crystal violet is composed of 2 solutions:

2.3.1.1 Two grams of Crystal violet was dissolved in 20 ml of 95% Ethanol.

2.3.1.2 Ammonium oxalate (0.8g) was dissolved in distilled water 80 ml.

The solution 1.1 and 1.2 were mixed homogeneously and stored in brown glass bottle.

2.3.2 Gram iodine: The iodine crystal 1.0 g was dissolved in 300 ml distilled water. Then 2g of potassium iodide was added slowly to the solution. The gram iodine solution was stored in a brown glass bottle.

2.3.3 Counter stain; Safranin o0 2.5g was dissolved in 95% Ethanol. The solution was diluted with 100 ml distilled water and also maintained in the dark brown bottle.

2.4 Instrument.

Gas Chromatograph. (GC)

2,4.1 Perkin Elmer Model F 17. Bacteriology and soil section Department of Agriculture. Ministry of Agriculture and Co-Operation.

2.4.2 Pye unicam series 104.

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Table 8. Operating conditions of the two gas chromatographs.

		Perkin Elmer model F 17	Pye unicam series 104
Column	type	Stainless steel	glass
	diameter (cm)	0.60	0.40
	length (m)	0.45	1.71
	packing	Porapak N	Porapak N
Oven	temp. (^{o}C)	55	90
Carrier gas		N_2	N_2
Flow rate (ml/min)		50	50
Detecter	type	FID	FID
	temp (^{o}C)	55	120

METHODS.

1. Preparation of rice root and rhizospheric soil samples.

The rice root and rhizospheric soil samples were collected ramdomly from the experimental plots in the form of intact soil core. At least 3 samples were collected for each treatment at each time interval. These intact soil cores were packed and transported in the ice-box. Sample were usually processed within 24 hours after arrival, otherwise they were stored in the cold room at 4-10°C.

1.1 Random sampling of the rhizospheric soil sample was taken from each soil core, pooled among similar treatment, and a duplicate amount of 0.1 g soil was enclosed in a 25 ml erlenmeyer flask containing either 5 ml NF medium or NF + 0.01% (W/V) yeast extract medium, pH 6.8, and stoppered by a suba-seal rubber stopper.

1.2 The remaining soil cores containing rice root were washed under stream of tap water until free of soil. These washed rice roots were divided into two parts.

1.2.1 Non-sterile root for studying nitrogen fixation activity of the rhizoplane and endorhizospheric bacteria. The washed rice roots were weighed 0.5 g and cut 2-3 cm long. Subsequently, the fragment of roots were placed in 25 ml erlenmeyer flask containing 5 ml of media; nitrogen-free (NF) medium or NF + 0.01% yeast extract (YE) medium, pH 6.8. The flasks were sealed with suba-seal rubber stopper.

1.2.2 Surface sterile root for studying nitrogen fixation activity by endorhizospheric bacteria. The washed roots were surface steriled by 70% ethanol for 3 min. and 2% chlorox for 20 min respectively. Then the sterile roots were rinsed three times with sterile water. Subsequently, they were cut 2-3 cm long and transferred into a 25 ml erlenmeyer flask containing either 5 ml NF medium or NF + 0.01% yeast extract medium pH 6.8 and sealed by the suba-seal rubber stopper.

2. ARA associated with rice root and rhizospheric soil.

All rice roots and rhizospheric soil samples were incubated in NF or NF + YE medium for 40 hours at room temperature (28°+ 2°C). Ten percent of the gaseous phase in each flask was removed and replaced with equal volume of acetylene. The flask was further incubated at room temperature for 1-8 hours and 0.2 ml (200 µl) of the gaseous mixture in the flask was examined in the gas chromatograph, Perkin Elmer F 17. Ethylene production per flask per hour was determined by comparing the peak height in cm with that of standard C_2H_4. The average dry weight of rice root and rhizospheric soil samples was used to report the ARA in µmol/g dry wt./hr.

Calculation of ARA associated with rice root and rhizospheric soil samples.

One ml of standard C_2H_4 was diluted to 2250 ml in air at 30°C, 1 atm. A 100 µl of this diluted standard C_2H_4 therefore contains 1.8×10^{-3} µmol C_2H_4 according to the calculation as follow :

100 µl standard gas (C_2H_4) in air = $\frac{100}{2250} \cdot \frac{273}{303} \cdot \frac{1}{22.4}$ µmol

= 1.8×10^{-3} µmol.

Usually, the attenuator 8 of the gas chromatograph (Perkin Elmer F 17) was used for this volume of standard gas injection, resulted in a peak height of 7.5 cm.

Example: For a rice root sample after 2 hours incubation, the peak height of a 100 µl injection was 8.4 cm at the attenuator 2. The gaseous volume of each incubation flask is 30 ml, therefore the µmol C_2H_4 produced per flask per hour is = 75.6×10^{-3}.

The dry weight of the root sample = 0.0714 g

Thus = 1.06 µmol C_2H_4/g/h

This ARA in µmol/g/h was converted into the N_2-fixing potential in kilogram N per hectare per day (kgN/ha/d) by assuming that,

(1) One mole of N_2 is reduced for every 3 mole of C_2H_2

(2) One hectare contain 1.6×10^5 rice hills.

(3) The act of acetylene reduction is linearly increased during 1-8 hours intervals.

Nitrogen fixing activity = A $\frac{\text{umol}}{\text{gxh}}$ x $\frac{\text{Bg}}{\text{hill}}$ x $\frac{24\ \text{h}}{\text{d}}$ x $\frac{1.6 \times 10^5 \text{hill}}{\text{ha}}$

x $\frac{\text{mole N}}{3 \times 10^6 \text{umole}}$ x 2.8×10^{-2} $\frac{\text{kg}}{\text{mole}}$

In practice, the dry weight of root in g/hill (B) was obtained from different experiments. (42)

3. Isolation of aerobic nitrogen fixing diazotrophic bacteria.

The aerobic N_2-fixing bacteria described in this paper was isolated from rice root and soil samples showing positive ARA.

The segments of non-sterile root and surface sterile root samples (4-5 pieces) in NF or NF + YE medium showing positive ARA in the previous experiments were placed on Dobereiner's NF plate and incubated at room temperature ($30\pm2^{\circ}C$) at least overnight. In the next morning, every plate was observed, and from each plate two-loopful of the bacteria grown in the vicinity of the root pieces were chosen according to the following criteria;

1. Intensive growth of bacteria around that root piece was observed after 1 day of incubation.
2. Difference in pigment production.

These two selected loopful of bacteria were transferred onto the left and right halves of the next NF plate respectively. This step was repeated 3-4 times until the presence of isolated colony was observed. Consequently two bacterial cultures were obtained from one starting plate. Homogeneous staining of bacteria cells within the colony was considered a pure culture.

Two-loopful of soil suspension in NF or NF + YE medium showing positive ARA per one flask were streaked onto the left and right halves of a Dobereiner's NF plate. In the next morning every plate was observed and one colony from each side (left and right) of the plate was chosen according to the following criteria:

1. The shortest time of incubation.
2. Difference in colonial morphology, such as pigment

production, sliming and translucence

These two selected colonies were transferred onto the left and right halves of the next NF plate respectively. This step was repeated 2-3 times until the presence of isolated similar colonies were observed. Appearance of identical colony on this NF medium and homogeneous staining of bacterial cells within the colony was considered a pure culture.

4. Screening for heterotrophic diazotroph having high N_2-fixing activity.

To check the nitrogen fixing ability of the pure culture afloopful of bacterial cell was transferred into 20 ml NF + 10% rich medium left at room temperature 24 hours. Cells were harvested by centrifugation a 1000 g. 10 min. The cell pellet was resuspended in NF medium and preincubated at room temperature for four hours. The flasks were sealed with suba-seal rubber stoppers. The gaseous phase was replaced with 10% C_2H_2 in air. Ethylene production was determined after 24 hours of incubation at room temperature by injecting a 200 µl of the gaseous phase into a gas chromatograph (Pye-Unicam series 104). The turbidity of this bacterial suspension was measured in a Spectronic 20 at 420 nm. The peak height in cm of C_2H_4 was converted into nmol by comparing with the peak height of known amount of standard C_2H_4. The ARA of the pure bacterial culture was reported in $\mu mol/OD_{420}/d$.

5. Observation of bacterial morphology.

5.1 The colonial morphology.

The isolated pure culture was streaked on Dobereiner's NF plate, pH 6.8 and incubated at room temperature. The plate was observed daily to record the days of incubation coinciding with the first appearance of colony formation. The colonial morphology was recorded according to Pelczar, Jr. and Chan, (43).

5.2 Gram stain (Huckler's modification)

The pure culture was smeared on slide, flame fixed and soaked in Gram crystal violet for 1 minute. Rinse the dye out with stream of tap water. Application of Gram iodine was further on the top for 1 minute and rinse once with water. Decolorize with 95% ethanol until the excess dye had been washed off. Wash slide again with water and drain. Then Safranin O was applied on this smeared area for 20 seconds and rinsed out with the tap water. The marked area was observed under light microscope (Olympus) at 1000 magnification to record Gram's staining.

5.3 Cell morphology.

By using the same slide prepared from Gram's staining, the cell morphology was observed at 1000 magnification according to Pelczar, Jr. and Chan, 1977 (43).