หอสมุดกลาง สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III.

RESULTS.

1. The influence of soil type on the N_2-fixing potential.

The non-fertillized plot (T_1=0,0,0,0) at Chumpae (low humic glay soil, pH 5.8) and Rangsit (Brackish water alluvil soil, pH 4.0), where similar rice varioty (RD. 7) was grown, were compared for their N_2-fixing potential profiles (Fig. 4). In the tillering stage (3 weeks after transplanting), the N_2-fixing potential associated with washed rice root (A) at Chumpae was 40-65 x 10^{-2} kgN/ha/d, which was 2-4 fold higher than that at Rangsit, 10-20 x 10^{-2} kgN/ha/d. Nontheless, the nitrogen fixing potential associated with washed root at Rangsit increased to a maximum of 60 x 10^{-2} kgN/ha/d in the ripening stage where as the N_2-fixing potential at Chumpae was maintained about the same level throughout the growing season. Application of yeast extract 0.01 % to NF media (NF + YE) enhanced the N_2-fixing potential at Chumpae to 120 x 10^{-2} kgN/ha/d in the tillering and maximum tillering stage but it did not show any significant effect at Rangsit.

Figure 4. The N_2-fixing potential in the non-fertilized plot (T_1=0,0,0,0) at Rangsit (Brackish water alluvial soil type, pH 4.0) and Chumpae. (low humic glay soil, pH 5.0)

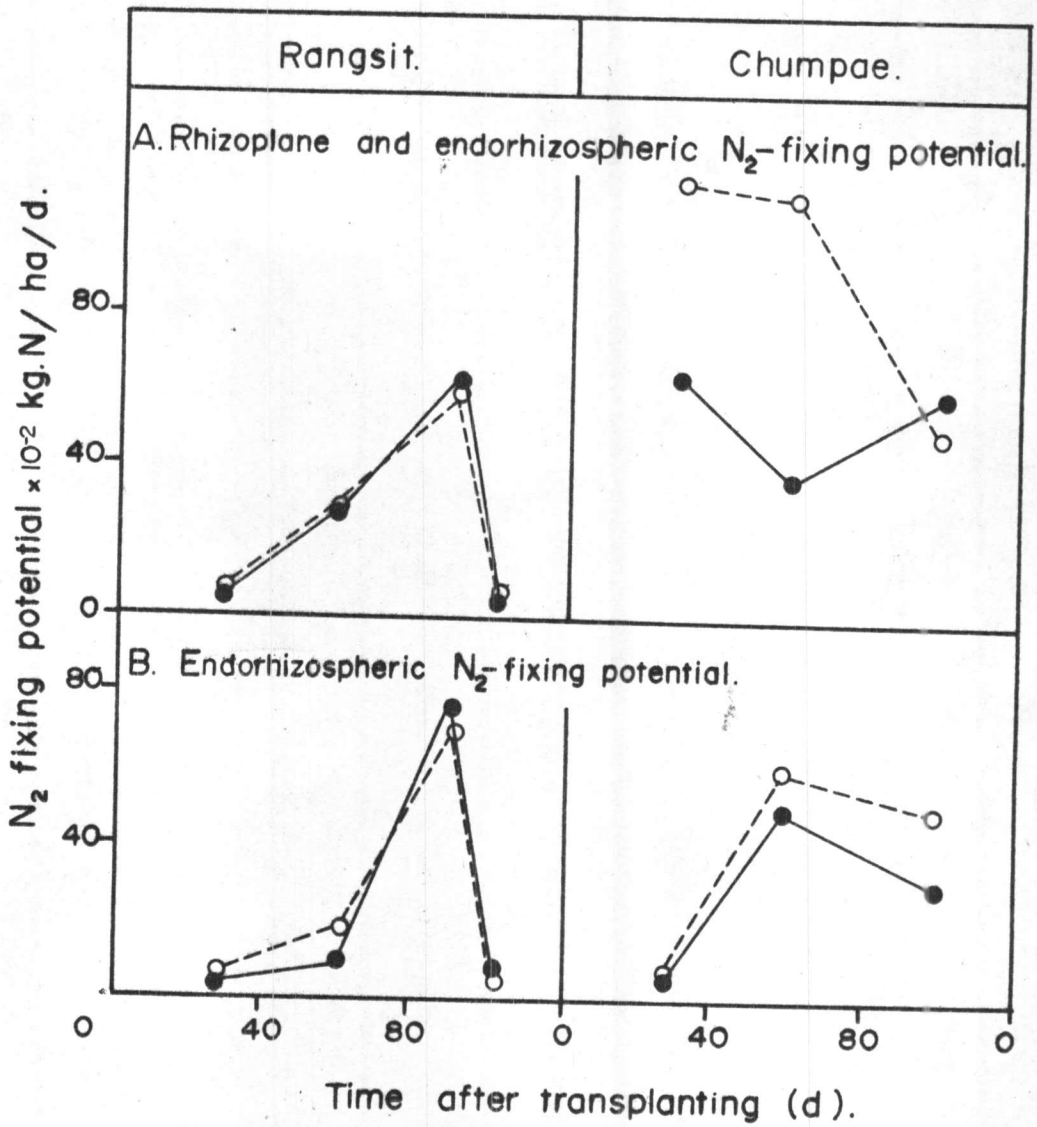
A. N_2-fixing potential associated with the washed rice roots containing rhizoplane and endorhizospheric diazotrophs.

B. N_2-fixing potential associated with the surface steriled roots containing only endorhizospheric diazotrophs.

●——● incubated in NF medium.

o----o incubated in NF + YE medium.

Each point represents the mean of 2 samples.


Rangsit.
Chumpae.
A. Rhizoplane and endorhizospheric N_2-fixing potential.
B. Endorhizospheric N_2-fixing potential.
N_2 fixing potential $\times 10^{-2}$ kg.N/ ha/d.
0
40
80
Time after transplanting (d).

The endorhizospheric N_2-fixing potential (B) at Rangsit showed the same profile as the rhizoplane+endorhizospheric N_2-fixing potential (A). The maximum potential was in the ripening stage. At Chumpae, different profile was observed. The N_2-fixing potential rose much earlier than at Rangsit and remained high towards ripening. Application of YE also enhanued the endorhizospheric N_2-fixing potential at Chumpae more than Rangsit. Although the rice roots were surface steriled, Figure 4 B showed that the endorhixospheric N_2-fixing potential profile was also affected by difference in soil type in the same way as in A.

Thus, the same variety of rice (RD.7) can show different N_2-fixing activity profile when grown in greatly different soil types.

2. <u>The influence of fertilizer application on the N_2-fixing potentail</u>.

2.1 Rangsit Rice Experiment Station.

There are three fertilized plots at Rangsit; T_5, T_6 and T_{10}. Twelve tens per ha of rice straw compost was applied in T_5, chemical fertilizer, with the ratio of N:P:K (50:25:25 Kg/ha) was applied in T_6, and T_{10} was the combination of both T_5 and T_6.

Comparing the N_2-fixing potential profiles of these three fertilized plots with T_1, the non-fertilized plot (Figure 5), it can be seen that they have nearly similar profiles. The maximum N_2-fixing potential associated with washed roots or surface sterile roots in the chemical fertilized plots (T_6) was significantly

Figure 5. The N_2-fixing potential in the non-fertilized and fertilized plot at Rangsit.

T_1 N,P,K, Compost = 0,0,0, kg/ha, 0 ton/ha.

T_5 N,P,K, Compost = 0,0,0, kg/ha,12 ton/ha.

T_6 N,P,K, Compost = 50,25,25,kg/ha, 0 ton/ha.

T_{10} N,P,K, Compost = 50,25,25,kg/ha,12 ton/ha.

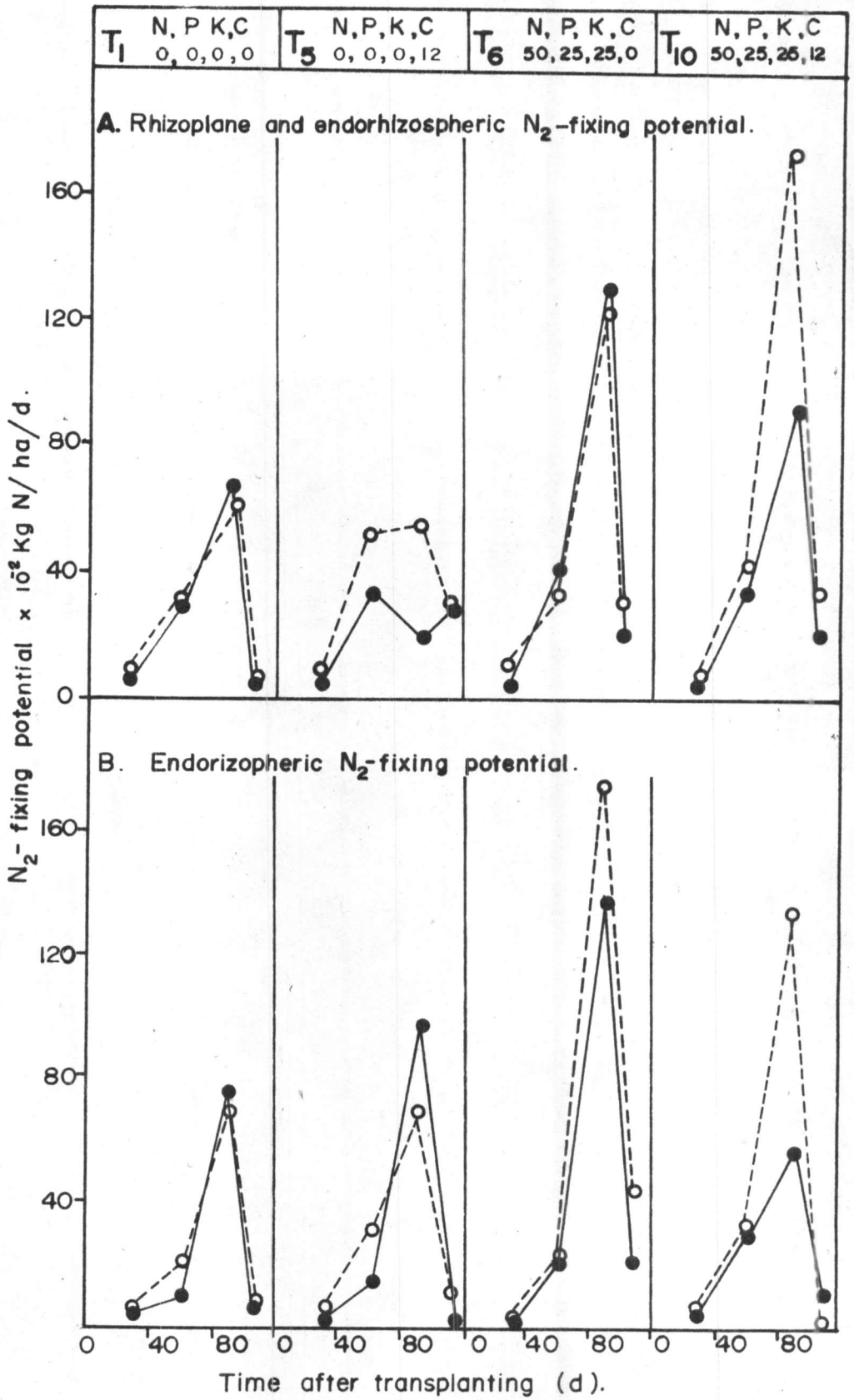
A Rhizoplane and endorhizospheric N_2fixing potential

B Endorhizospheric N_2fixing potential.

●——● incubated in NF medium

o------o incubated in NF + YE medium

Each point represents the mean of 2 samples


T1 N, P K, C 0, 0, 0, 0
T5 N, P, K, C 0, 0, 0, 12
T6 N, P, K, C 50, 25, 25, 0
T10 N, P, K, C 50, 25, 25, 12
A. Rhizoplane and endorhizospheric N2-fixing potential.
B. Endorizopheric N2-fixing potential.
N2- fixing potential x 10² Kg N/ ha/ d.
0
40
80
120
160
Time after transplanting (d).

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higher than in the non-fertilized plots (T_1). Application of organic fertilizer (T_5) seemed to be less effective than the chemical fertilizer, because the maximum endorhizospheric N_2-fixing potential was more or less the same as in T_1 . The addition of yeast extract into NF media during preincubation enhanced the N_2-fixing potential associated with the rice root grown in the most fertilized plot (T_{10}) only at the ripening stage.

Tentatively, the application of either organic or chemical fertilizer does not inhibit the N_2-fixing potential contributed by enderhizospheric and rhizoplane bacteria, however addition of chemical fertilizer seemed to have more pronuonced effect on the maximum activity.

2.2 Chumpae Rice Experiment Station.

Comparison of the N_2-fixing potential profiles between the two fertilized plots and the non-fertilized plot (T_1) was shown in Figure 6. The Application of NPK or only P and K fertilizers did not change the N_2-fixing potential profiles associated with the washed root (Fig.6A). but different effects was observed for the enderhizospheric N_2-fixing potential profiles (Fig. 6B).

The chemical fertilzer applied in T_2 and T_5 enhanced the N_2-fixing potential associated with the washed root especially in the vegetative phase. Obviously, the application of NPK fertilizer (T_5) resulted in higher rhizoplane N_2-fixing potential than the application of only P and K fertilizer (T_2) at every

Figure. 6 The N_2-fixing potential in the non-fertilized and fertilized plot at Chumpae.

T_1 N,P,K, = 0,0,0. kg/ha

T_2 N,P,K, = 0,38,25. kg/ha

T_5 N,P,K, = 113,38,25 kg/ha

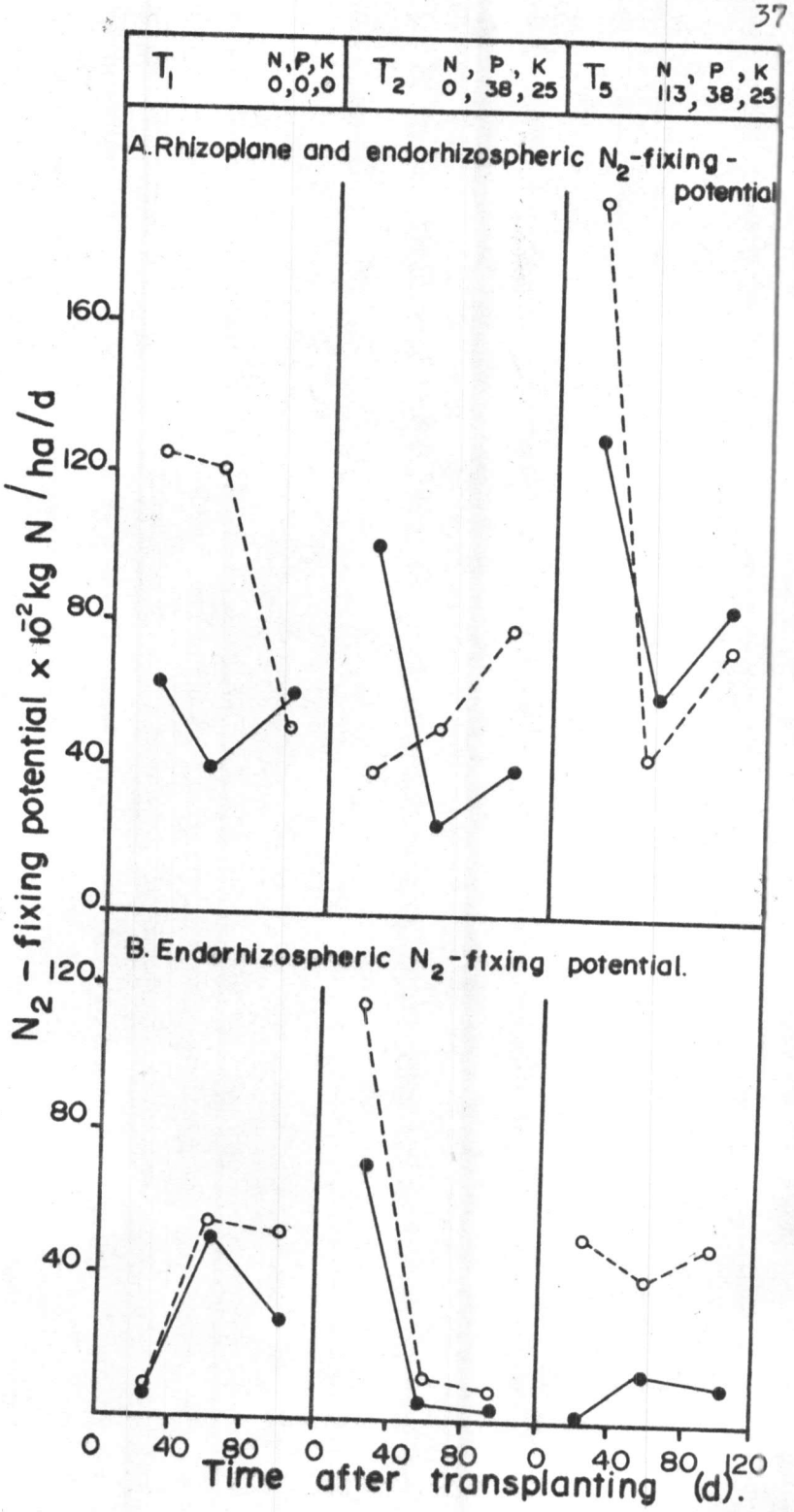
A Rhizoplane+endorhizospheric N_2fixing potential

B Endorhizospheric N_2-fixing potential.

●——● incubated in NF medium

o------o incubated in NF + YE medium

Each point represents the mean of 2 samples


T1 N,P,K 0,0,0
T2 N, P, K 0, 38, 25
T5 N, P, K 113, 38, 25
A. Rhizoplane and endorhizospheric N2-fixing potential
B. Endorhizospheric N2-fixing potential.
N2 - fixing potential x 10-2kg N /ha/d
0
40
80
120
160
Time after transplanting (d).

stage of rice growth (Fig. 6A).

No uniform prefile was observed with the endorhizospheric N_2-fixing potential (Fig. 6B), however application of NPK seemed to inhibit the endorhizospheric N_2-fixing potential.

Addition of YE in the NF medium during preincubation showed inconsistent effect, however stimulating effect was more frequently observed.

In conclusion the results at Rangsit and Chumpae showed that the application of either chemical fertilizer or organic matter or both types of fertilizer did not change the N_2-fixing potential profiles. Different types of fertilization may cause variable values of the maximum N_2-fixing potential. Application of the N - fertilizer at the rate as high as 100kg/ha or over tends inhibit the enderhizospheric N_2-fixing potential.

3. <u>The influence of rice variety on N_2-fixing potential</u>.

At Tapra, North-Eastern Agricultural Bureau, two non-sticky rice varieties (RD. 15 and Kowmali) and 2 sticky rice varieties (RD. .6 and Sanpatong) were grown. Their N_2-fixing potential profiles throughout one rice growing season were shown in Figure 7

3.1 The non-sticky rice varieties (RD . 15 and Kowmali).

The N_2-fixing potential profiles associated with the 2 non-sticky rice varieties were similar. The peak of N_2-fixing potential was observed in the tillering stage with the maximum (40×10^{-2} kgN/ha/d) about 40 days after transplanting (DAT).

Figure 7. The N_2-fixing potential associated with non-sticky rice (RD.15, Kowmali) and sticky rice (RD.6, Sanpatong)

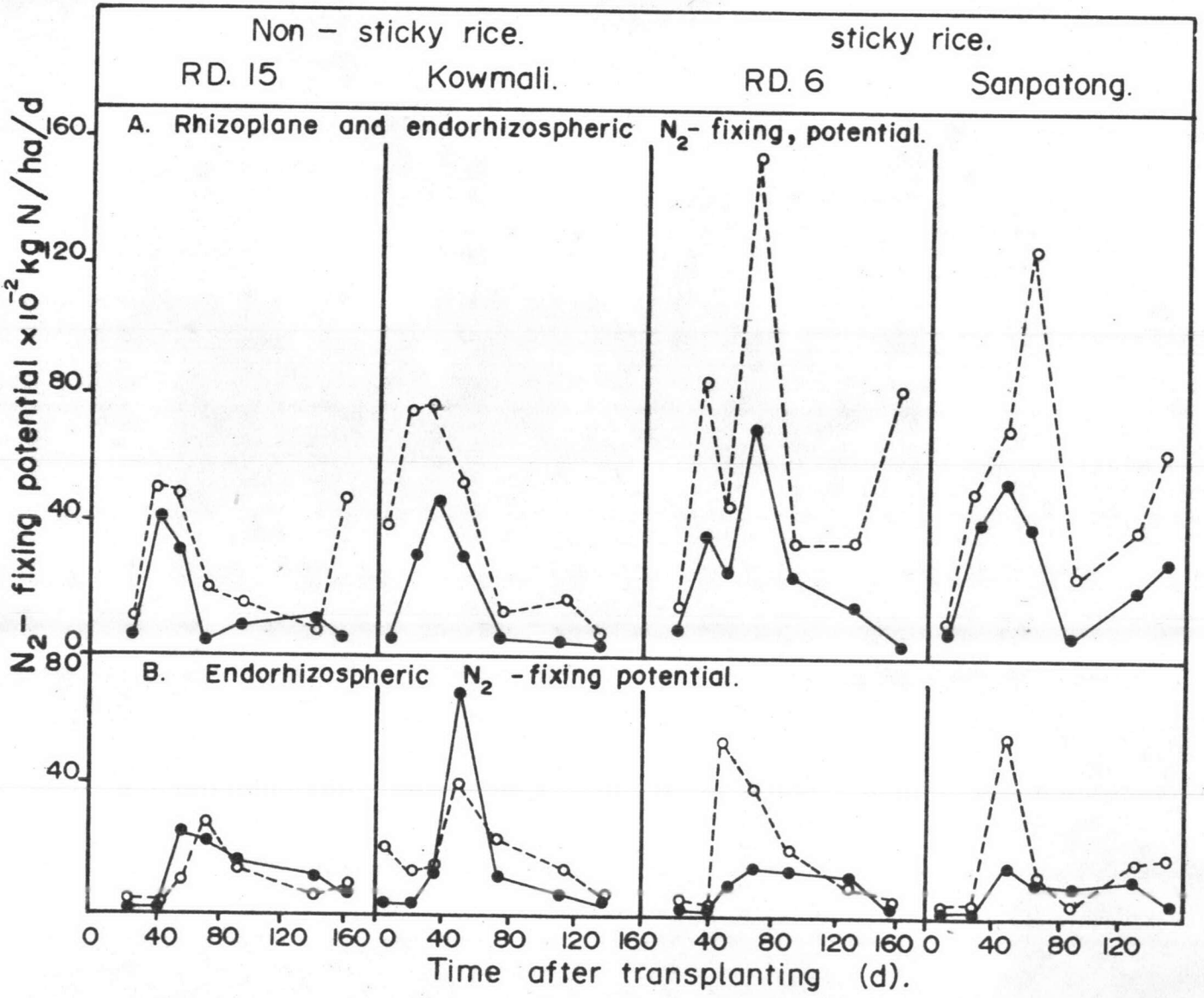
A Rhizoplane and endorhizospheric N_2-fixing potential.

B Endorhizospheric N_2-fixing potential.

●——● incubated in NF medium

o------o incubated in NF + YE medium

Each point represents the mean of 2 samples


Non – sticky rice.
sticky rice.
RD. 15
Kowmali.
RD. 6
Sanpatong.
A. Rhizoplane and endorhizospheric N_2- fixing, potential.
B. Endorhizospheric N_2 -fixing potential.
N_2- fixing potential $\times 10^{-2}$ kg N/ha/d
0
40
80
120
160
Time after transplanting (d).

After 80 DAT, the N_2-fixing potential clearly decreased and was at the level of 10 x 10^{-2}kgN/ha/d towards the ripening stage. Addition of YE during proincubation enhanced N_2-fixing potential slightly. The rhizoplane and endorhizospheric N_2-fixing potential associated with Kowmali seemed to be higher than RD.15, because the area under peak at the tillering stage was greater. The data indicated that the difference in varieties of the non-sticky rice did not affect the N_2-fixing potential profile, but the old variety, Kowmali seemed to show higher rhizospheric N_2-fixing potential than the new variety, RD.15.

3.2 The sticky rice varieties (RD.6 and Sanpatong)

The N_2-fixing potential profiles associated with the 2 sticky rice varieties were also similar showing the peak of high activity in the tillering stage (40-70x10^{-2} kgN/ha/d) and decreased by 80 DAT to about 20-30x10^{-2}KgN/ha/d towards harvesting. The application of YE to NF ' medium clearly enhanced the N_2-fixing potential in both varieties upto more than 120x10^{-2}kgN/ha/d. This effect was also observed in the endorhizospheric activity associated in the surface steriled roots (Fig. 7B).

These results indicated that the difference in variety of the two sticky rice did not affect the N_2-fixing potential profile, the old variety Sanpatong and the new variety, RD.6 showed the same level of N_2-fixing potential but application of YE seemed to enhanced the potential associated with RD.6 more than Sanpatong.

3.3 Comparison of the N_2-fixing potential between the non-sticky rice, and the sticky rice varieties.

Although there is no difference in the N_2-fixing potential profiles among the four rice varieties grown at Tapra, but the values of the N_2-fixing potential of the 2 sticky rices studied were quite higher than the 2 non-sticky rices.
The application of YE during preincubation enhanced the N_2-fixing potential of the 2 sticky rices much more than the 2 non-sticky rices studied.

4. The evaluation of total N_2-fixing potential per crop.

An attempt to evaluate the total amount of nitrogen fixing potential by the rhizospheric bacteria in kg N/ha/crop was carried out by cutting out the areas under peaks of the N_2-fixing potential profiles from the figure 5,6, and 7 and weighed in a four decimals Balance Mettler (H^{10} Tw) assuming that the paper weight was homogeneous.

According to the scales used in these figures, the weight of paper, 0.0161 g was equivalent to 16 kg nitrogen fixed per hectare.

The total nitrogen fixing potential per hectare per crop was presented in figure 8. When the rice root samples were incubated in NF medium, the total nitrogen fixing potential contributed by the rhizospheric bacteria at the three sites was in the range of 19-72 kgN/ha/crop. Supplementation with YE to the NF medium enhanced the nitrogen fixing potential of the rhizospheric bacteria in 82 % of the samples to the range of 30-89 kgN/ha/crop.

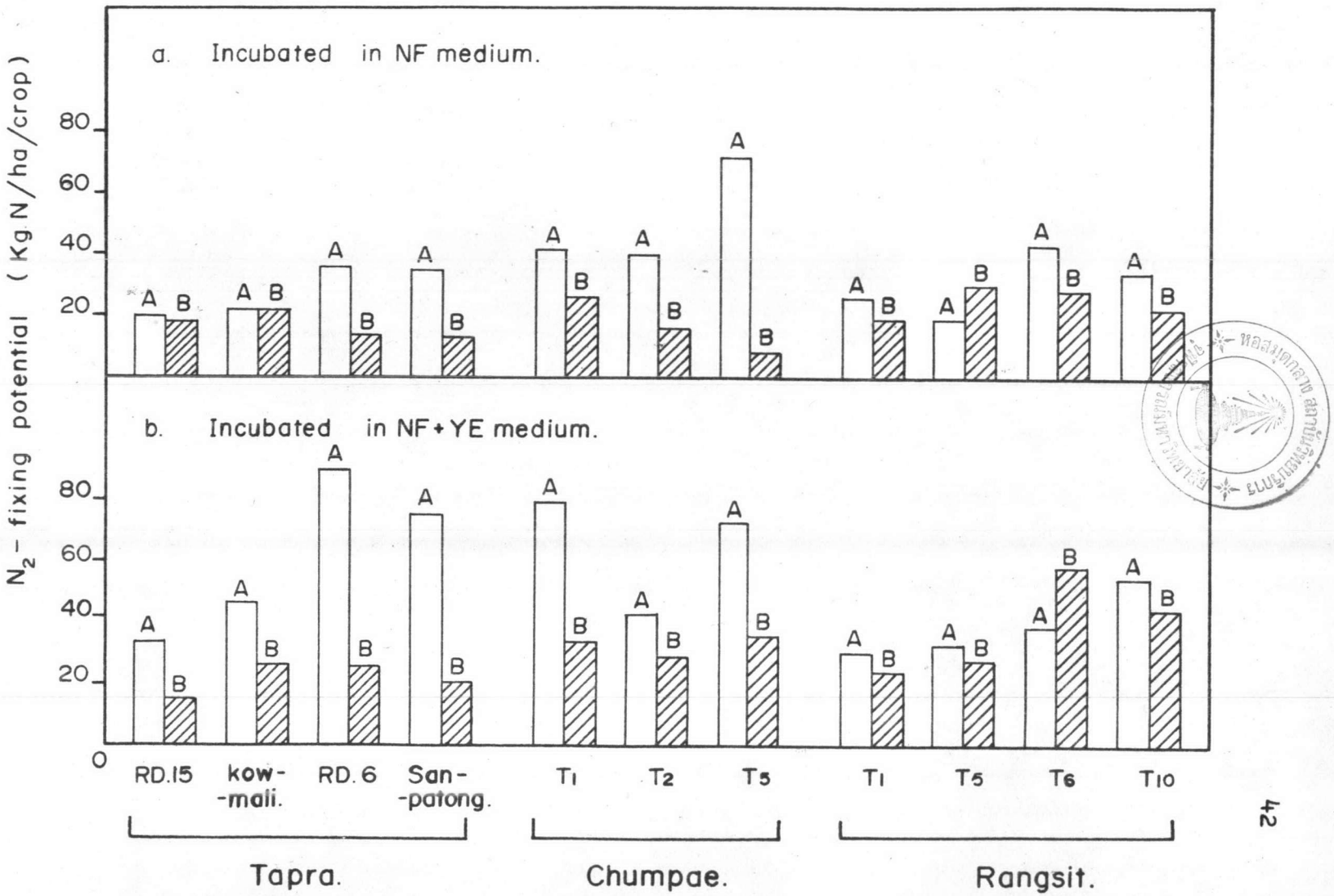
Figure 8. The evaluation of total nitrogen fixed by rhizospheric bacteria per hectare per crop.

a. Incubated in NF medium

b. Incubated in NF + yeast extract medium,

The amount of nitrogen fixed by the rhizoplane + endorhizospheric bacteria.

The amount of nitrogen fixed by the endorhizospheric bacteria only.


a. Incubated in NF medium.
b. Incubated in NF+YE medium.
N_2 - fixing potential (Kg N/ha/crop)
0
20
40
60
80
A
B
RD.15
kow--mali.
RD.6
San--patong.
T1
T2
T5
T1
T5
T6
T10
Tapra.
Chumpae.
Rangsit.

5. The morphology of isolated pure bacterial culture.

Two hundred fifty nine pure bacterial cultures were isolated from the rice root and rhizospheric soil samples showing positive C_2H_2 reduction activity as described in Methsods. They were examined for the colonial morphology on NF plate, pH 6.8 in the aerobic condition. The cell structure after staining was observed under light microscope at 1000 magnification. Their colonial and cellular morphology, and the rate of colony formation were summarized in Table 9, 10, 11 and 12.

5.1 The morphology of the pure bacterial cultures isolated from Chumpae. There were 33 cultures isolated from this experimental site as shown in Table 9, most of them (90%) were the 1 day fast growing bacteria. Generally these colonies formed circular shape and pin-point size on acrobic NF plate with no pigment production. All of them showed negative Gram's stain and short rod structure under microscopic examination.

5.2 The morphology of the pure cultures isolated from Rangsit. Table 10 showed the morphology of 43 cultures from Rangsit, most of them formed colony on the second day of incubation (67%), only 13 cultures or 30% were the 1 day fast growing bacteria. Generally, their colonial morphology were circular in shape and pin-point in size. They were slimy and could not produce pigment. Microscopic examination showed that all of them were negative to Gram's stain, and the major cellular structure (50%) were rodlike.

Table 9. The morphology of the bacterial cultures isolated from rice rhizosphere at Chumpae.

The aerobic nitrogen fixing diazotrophic bacteria were isolated from the rice root and soil samples as described in Methods.

Total number isolated.	Incubation time (d)	Number of bacteria (%)	Colonial morphology.			General infor-mation.	Cell structure *				
			general structure.	no sliming (%)	Opaque (%)		pin-point (%)	short rod (%)	rod (%)	long rod (%)	round rod (%)
33	1	30 (91)	circular smooth rim elevate no-pigment translucent sliming pin-point size	1 (3)	11 (33)	Gram- single	5 (15)	6 (49)	6 (18)	2 (6)	1 (3)
	2	3 (9)	"	—	—	"	—	1 (3)	1 (3)	1 (3)	—
							5 (15)	17 (52)	7 (21)	3 (9)	1 (3)

* 1000 magnification

Table 10. The morphology of the bacterial cultures isolated from rice rhizosphere at Rangsit

The aerobic nitrogen fixing diazotrophic bacteria were isolated from the rice root and soil samples as described in Methods.

Total number isolated	Incubation time (d)	Number of bacteria	Colonial morphology			general information	Cell structure*				
			general structure	no sliming (%)	Opaque (%)		pin-point (%)	short rod (%)	rod (%)	long rod (%)	round rod (%)
43	1	13 (30)	circular smooth rim elevate no pigment translucent sliming pin point size	2 (5)	–	Gram- single	5 (12)	3 (7)	5 (12)	–	–
	2	29(67)	,,	–	–	,,	–	9 (21)	19(44)	1 (2)	–
	3	1 (2)	,,	–	–	,,	–	–	–	1 (2)	–
							5 (12)	12 (28)	24 (56)	2 (4)	

* 1000 magnification

5.3 The morphology of the bacterial cultures isolated from Tapra. A total of 183 pure bacterial cultures were isolated from Tapra samples, of which 95 were from the non-sticky rice, RD.15 and Kowmali. The morphology of these bacterial cultures were summarized in Table 11. Most of the bacteria isolated, from the new rice variety, RD.15 formed colonies after they were incubated for only one day (70%), whereas only 50% of the bacteria from the old rice variety, Kowmali appeared as colonies after 1 day of incubation. It can be seen that about 50% of the bacteria isolated from Kowmali required 2-4 days for colony formation, so called slow-growing bacteria. In the new variety, RD. 15 only 30% of the bacteria isolated were the slow-growing type. However no obvious difference in the colonial and cellular morphology were observed between these two rice varieties, their colonics were slimy, circular and pin-point in size, and contained no pigment. All of them were Gram negative. The major cell structure was red shape, only a few were pin-point.

Table 12 showed the morphology of the bacteria isolated from sticky rice altogether 88 pure cultures, 46 from RD.6 and 42 from Sanpatong. Most of the bacteria isolated from the new rice variety, RD. 6 appeared as colonics within 1 day of incubation (63%), whereas only 54% of the bacteria isolated from the old rice variety, Sanpatong appeared as colonics on the same day. About 45% of bacteria isolated from the old variety and 37% of bacteria isolated from the new variety were the slow growing type requiring 2-4 days of incubation to form colony.

Table 11. The morphology of the bacterial cultures isolated from rhizospheric non-sticky rice at Tapra.

The aerobic nitrogen fixing diazotraphic bacteria were isolated from rice root and soil sample of the two non-sticky rice varieties: RD. 15 (modern variety) and Kowmali (old variety) as described in Methods.

Rice Variety	Total number isolated	Incubation time (d)	Number of bacteria	colonial morphology				general information	cell structure *				
				general structure	no sliming (%)	Opaque (%)	1-3 mm size (%)		pin point (%)	short rod (%)	rod (%)	long rod (%)	round rod (%)
RD. 15	48	1	34 (71)	circular smooth rim elevate sliming translucent pin-point no-pigment	3 (6)	—	2 (4)	Gram- Single	9 (19)	5 (10)	18 (38)	2 (4)	—
		2	13 (27)	,,	2 (4.)	1 (2.)	1 (2.)	,,	2 (4)	3 (6)	8 (17)	—	—
		4	1 (2)	,,							1 (2)		
Kowmali	47	1	22 (47)	,,	4 (9)	1 (2)	8 (17)	,,	—	5 (11)	11 (23)	3 (6)	3 (6)
		2	24 (51)	,,	2 (4)	1 (2)	1 (2.)	,,	2 (4)	7 (15)	13 (28)	1 (2)	1 (2)
		4	1 (2)	,,									1 (2)

* 1000 magnification.

Table 12. The morphology of the bacterial cultures isolated from rhizospheric sticky rice at Tapra.

The aerobic nitrogen fixing diazotrophic bacteria were isolated from rice root and soil samples of the two sticky rice varieties: RD. 6 (modern variety) and Sanpatong (old variety) as described in Methods.

Rice Variety	Total number isolated	Incubation time (d)	Number of bacteria	Colonial morphology				general information	cell structure *				
				general structure	no sliming (%)	Opaque (%)	1-3 mm size (%)		pin-point (%)	short rod (%)	rod (%)	long rod (%)	round rod (%)
RD.6	46	1	29(63)	circular smooth rim elevate sliming translucent pin-point no pigment	5 (11)	4 (9)	3 (7)	G-single	5 (11)	9 (20)	9 (20)	3 (7)	3 (7)
		2	13 (28)	,,	3 (7)	2 (4)	1 (2)	,,	1 (2)	3 (7)	6 (13)	1 (2)	2 (4)
		4	1 (2)	,,	—	—	—	,,	—	—	1 (2)	—	—
		5	3 (7)	,,			3 (7)				3 (7)		
Sanpa-tong.	42	1	23(55)	,,	3 (7)	2 (5)	4 (10)	,,	6 (14)	4 (10)	10 (24)	2 (5)	1 (2)
		2	17 (41)	,,	5 (12)	1 (2)	1 (2)	,,	5 (12)	4 (10)	7 (17)	—	1 (2)
		4	2 (5)	,,	—	—	—	,,		1 (2)	1 (2)	—	—

* 1000 magnification.

The general colonial morphology on NF plate of the isolated bacteria from both rice varieties were slimy circular shape, with the pin point size and no pigment production. All of them were Gram negative, rod ahape. The rice plant of any variety grown at Tapra seemed to have many different types of diazotrophic bacteria associated with the rhizospher Comparing to Chumpae and Rangsit, since the distribution in incubation time required for colony observation varied from 1-5 days. Unlike Tapra, the bacteria isolated from Chumpae mostly formed colony after only one day of incubation, indicating the homogeneity of the bacterial population.

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

All the pure bacterial cultures, isolated from rice root and rhizospheric soil samples were tested for their N_2-fixation ability by C_2H_2 - reduction assay as described in Methods. Figure 9 showed the histograms of the number of aerobic N_2-fixing bacteria at different levels of ARA which are 1-99, 100-499, 500-999 and higher than 1000 nmol/OD_{420}/d respectively. The bacteria selected for further study were these having ARA higher than 1,000 nmol or 1 nmol /OD_{420}/d. According to this criteria, twelve cultures were chosen from Tapra and 5 each from Chumpae and Rangsit. Among these 22 pure cultures having the ARA ranging from 1 - 6 umol /OD_{420}/d, eight cultures representing different morphology and site of collection were reselected for further chracterization.

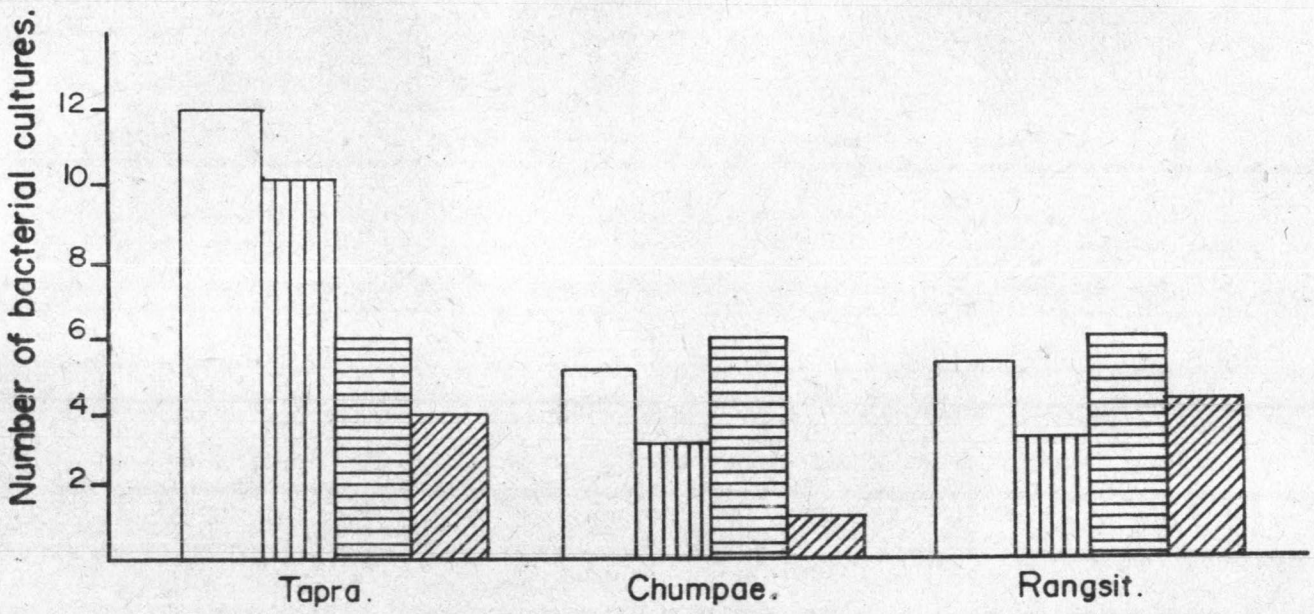


Figure 8. The histograms of the number of isolated bacterial cultures from different areas screened by the difference in the level of ARA.

□	ARA	>	1000	nmol	/ OD_{420} / d.
▥	500	<	ARA ≤	1000	"
▤	100	<	ARA ≤	500	"
▨	ARA	≤	100		"

7. Characterization of the aerobic diazotrophic bacteria.

7.1 The effect of temperature on bacterial growth.

The eight bacterial cultures chosen were grown in NF + 10 % rich medium. The cells were divided in 2 parts and harvested at the stationary phase by centrifugation. The packed cells in the first part was resuspended in NF medium and the second part in NF + 10% rich medium, pH 7.0. A proper aliquot was taken and diluted to the initial OD_{420} of 0.02 in each tube of either medium. Triplicate tubes were incubated at 25, 30, 37, 42, 46, 50°C for five hours, and the turbidity manifested with bacterial growth was observed in a Spectronic 20 at 420 nm.

The effect of variation (25-30°C) in temperature on growth in NF and NF + 10% rich media for five hours was shown in Figure 10. According to the plot of OD_{420} versus temperature in NF + 10% rich medium, the aerobic diazotrophic bacteria were classified into three groups. Group A showed the optimum temperature for growth at 37°C inwhich the difference of growth between 37°C and 30°C was quite drastic about 0.4 OD_{420} unit. Group B, although showed the optimum temperature at 37°C like Group A, but the difference of OD_{420} at 37°C and 30°C was only 0.2 OD_{420} unit or less. Group C was distinquished by the optimum temperature for growth between 42-46°C.

All the eight bacterial cultures could not grow in the NF liquid medium after the five hours of incubation at any temperature.

Figure 10. The effect of temperature on the growth of aerobic diazotrophs.

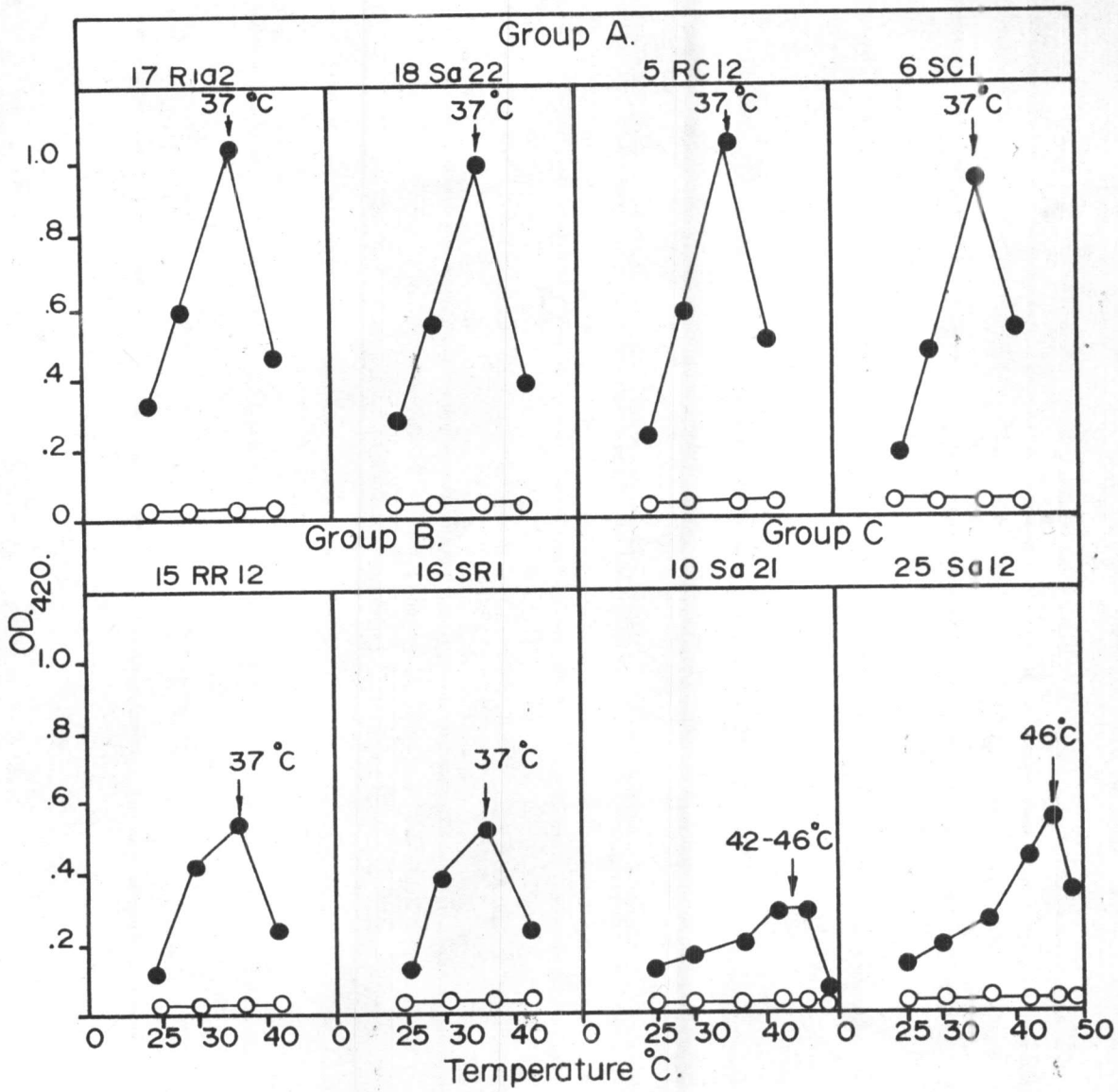
The eight bacterial cultures chosen were grown in NF + 10 % rich medium. The cells were divided in 2 parts and harvested at the stationary phase by centrifugation. The packed cells in the first part was resuspended in NF medium and the second part in NF + 10 % rich medium, pH 7.0 A proper aliquot was taken and diluted to the initial OD_{420} of 0.02 in each tube of either medium. Triplicate tubes were incubated at 25,30,37,42,46,50°C for five hours, and the tubidity manifested with bacterial growth was observed in a spectronic 20 at 420 nm.

O———O incubated in NF medium

●———● incubated in NF + 10 % rich medium

Each point represents the mean of 3 samples.

The original information of these eight characterized bacterial cultures was summarized in the Appendix (Table 14).


Group A.
17 R1a2
18 Sa 22
5 RC 12
6 SC1
37 °C
Group B.
15 RR 12
16 SR1
Group C
10 Sa 21
25 Sa 12
42-46°C
46°C
OD420.
1.0
.8
.6
.4
.2
0
25
30
40
50
Temperature °C.

7.2 The effect of pH of the medium on bacterial growth.

A bacterial culture grow in NF + 10% rich medium at pH 7.0 to stationary phase was used as starter. After dividing cells into 2 parts, cells were collected by centrifugation, the first part was resuspended in NF medium at different pH. The second part was resuspended in NF + 10% rich medium at different pH. The initial OD_{420} in each tube was adjusted to 0.02 by dilution. Triplicate tubes at each pH value of each medium were incubated at 37°C for 5 hours. Bacterial growth was observed by measuring turbidity at 420 nm. The final pH after 5 hours of incubation was observed by pelleting the cells and measured the pH of the supernatant liquid.

Variation of pH (4.5-7.5) caused different effect on growth of the eight bacterial cultures. Figure 11 showed the plot of OD_{420} versus initial pH of the medium of each bacterium. The arrow indicated the shift in pH after 5 hours of incubation. All the 4 bacteria classified in Group A by the previous experiment showed the same patterns, this is also true with the other two group B and C.

In NF + 10% rich medium Group A preferred the slightly basic and neutral pH and showed the optimal pH at 7.5. However, the growth occured quite well in slightly acidic media (pH 4.5 5.5), the difference of OD_{420} after 5 hours of incubation was less than 0.1 OD unit. Group B, was similar to group A, by having the optimal initial pH of 7.5, but the decreasing of the initial pH

Figure 11. The effect of pH on the growth of aerobic diazotrophs. A bacterial culture grown in NF + 10 % rich medium to stationary phase was used as starter. After dividing cells into 2 parts, cells were collected by centrifugation, the first part was resuspended in NF medium at different pH. The second part was resuspended in NF + 10 % rich medium at different pH. The initial OD_{420} in each tube was adjusted to 0.02 by dilution. Triplicate tubes at each pH value of each medium were incubated at 37°C for 5 hours. Bacterial growth was observed by measuring turbidity at 420 nm. The final pH after 5 hours of incubation was observed by pelleting at cells and measured the pH of the supernatant liquid.

O———O incubated in NF medium

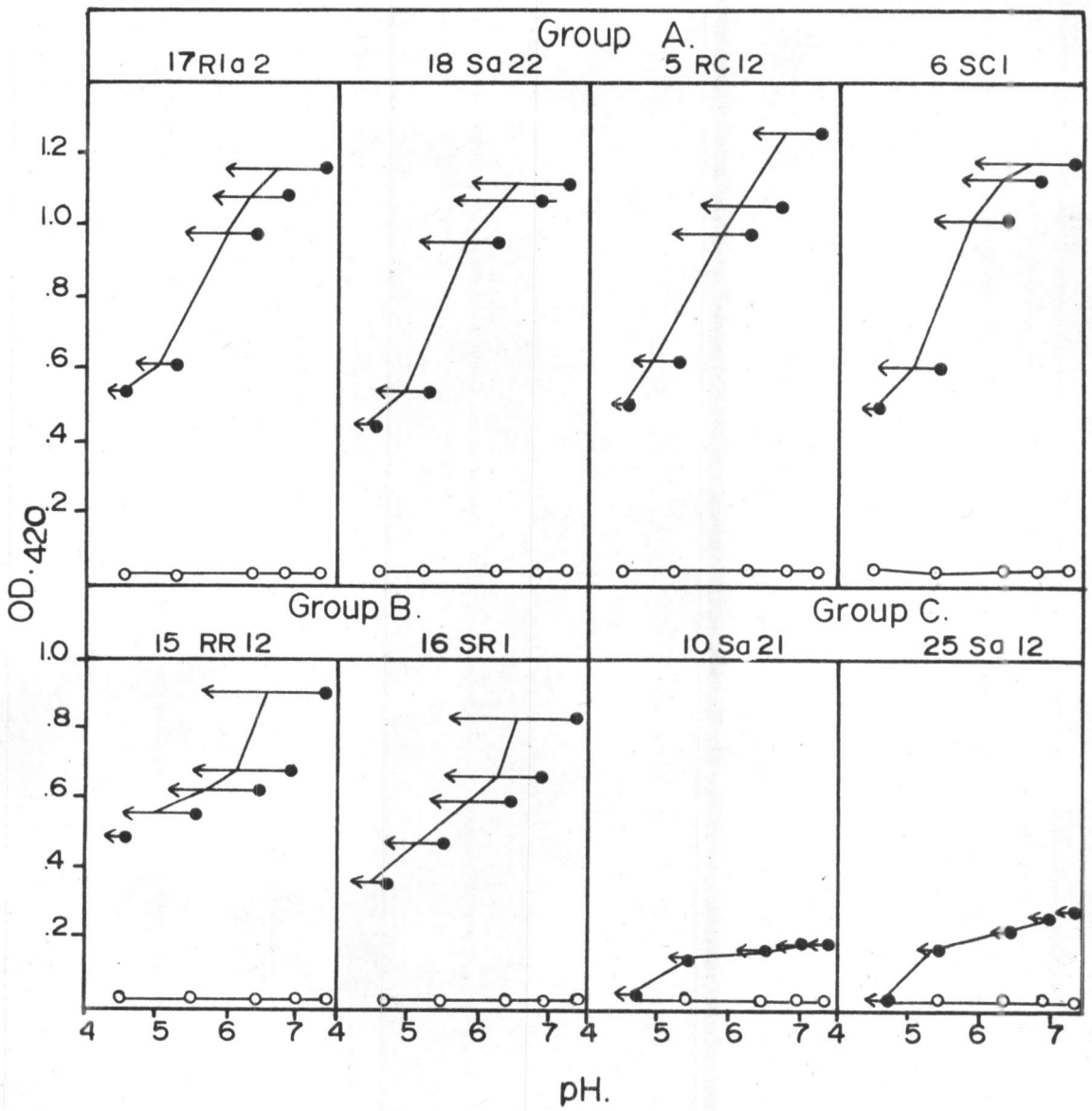
●———● incubated in NF + 10 % rich medium

final pH after 5 hrs. of incubation.

initial pH before incubation.

Each point represents the mean of 3 samples.

The original information of these eight characterized bacterial cultures was summarized in the Appendix (Table 14).


Group A.
17 R1a 2
18 Sa 22
5 RC 12
6 SC 1
Group B.
15 RR 12
16 SR 1
Group C.
10 Sa 21
25 Sa 12
OD. 420
1.2
1.0
.8
.6
.4
.2
4
5
6
7
pH.

to 7.0 resulted in a significant decreasing in growth by 0.3 OD unit. Thus Group B is more sensitive to neutral and slightly acid pH than Group A. Again, Group C was obviously characterized by the very low growth even in NF + 10% rich media at any initial pH. Variation of initial pH of the media (5.5-7.5) did not have much effect on growth. Besides, the pH shift after 5 hours of incubation (←———) were very small comparing to the other two groups. Figure 10 also showed that all of them produced acidic compounds in NF + 10% rich media, causing the drop of pH after five hours of incubation. None of them grew in NF medium at any initial pH.

7.3 Relationship between N_2-fixing activity and stage of growth.

From the difference in patterns of temperature response and pH response, there seemed to be three catagories (A,B, and C) of diazotrophic bacteria. In the next experiment, we selected three bacterial cultures (one of each group). The growth patterns in NF + 10% rich medium at 37°C was followed. Figure 12 showed the logarithmic growth curves of the representative bacteria from Group A (17R1a2), Group B (15RR12) and Group C (25Sa12). The time required to reach midlog phase, which is the most active phase of each bacteria was 3, 3.5 and 4.5 hours for Group A, B, and C respectively. The maximum growth of Group C was as less as a quarter of Group A or B at the stationary phase. From this information, we can prepare the bacterial cultures in its midlog age for studying

Figure 12. The growth curve of the nitrogen fixing bacteria in NF + 10 % rich medium, pH 7.0 at 37°C

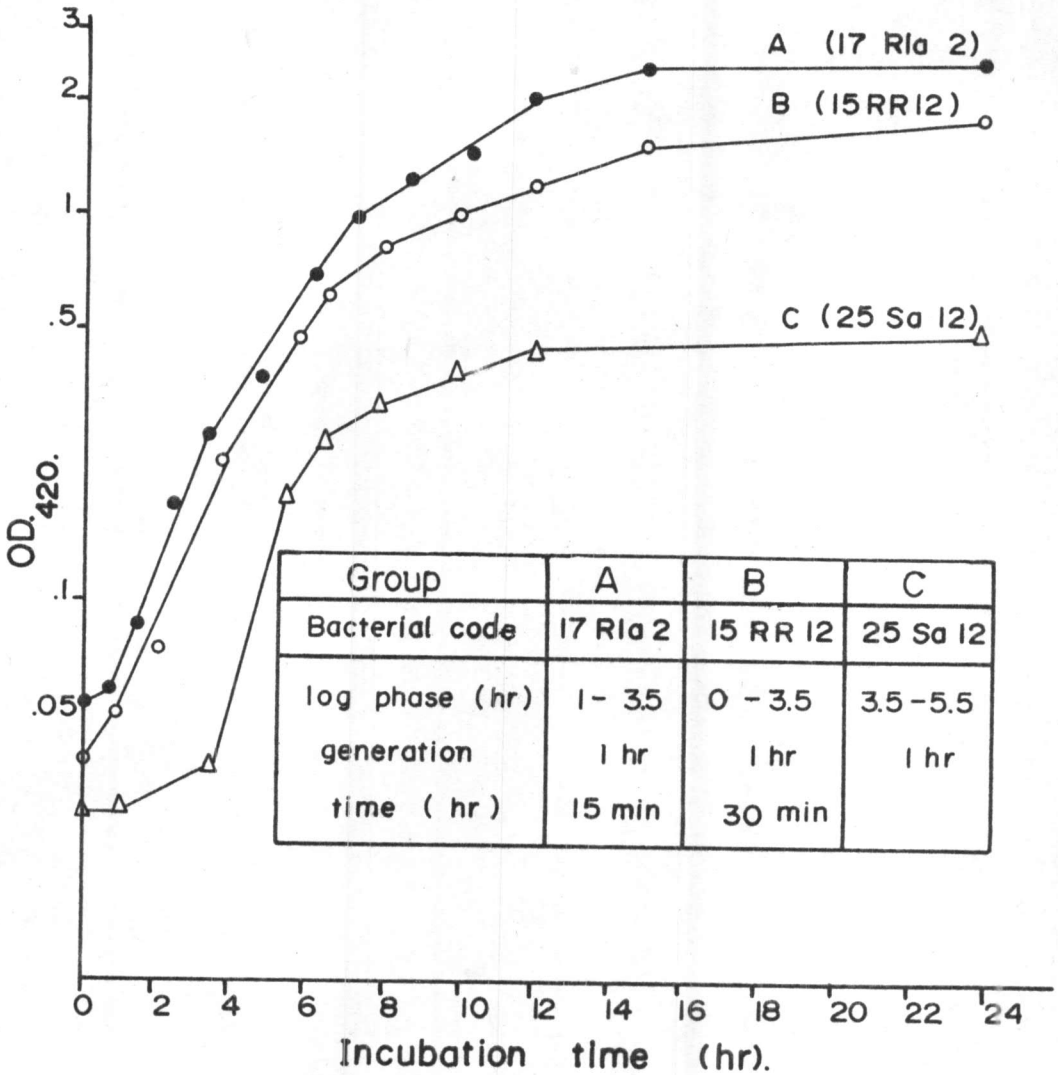
●——● The representative bacteria from Group A.

O——O The representative bacteria from Group B.

△——△ The representative bacteria from Group C.

Each point repersents the mean of 3 samples.

The original information of the selected three bacterial cultures was summarized in the Appendix (Table 14).



Group	A	B	C
Bacterial code	17 Rla 2	15 RR 12	25 Sa 12
log phase (hr)	1 - 3.5	0 - 3.5	3.5 - 5.5
generation time (hr)	1 hr 15 min	1 hr 30 min	1 hr

the correlation between growth and ARA in NF medium.

The bacterial cells harvested in the midlog phase were suspended in NF medium. Each culture was divided into 14 identical tubes with the starting concentration of 0.52 OD_{420}. The first two tubes were assayed for ARA at 0 time. The rest were incubated at room temperature (29-30°C). At various time intervals, a duplicate of samples were analyzed for ARA and OD_{420}. As shown in Figure 13, bacteria from Group A showed maximum ARA about 52 nmol/OD/h at 12 hours of incubation which was in the early stationary phase. On the contrary, bacteria from Group B showed the maximum ARA about 75 nmol/OD/h at 8 hours of incubation, which was midlog phase. The bacteria from Group C, which always showed remarkedly low OD_{420} failed to show ARA under this condition.

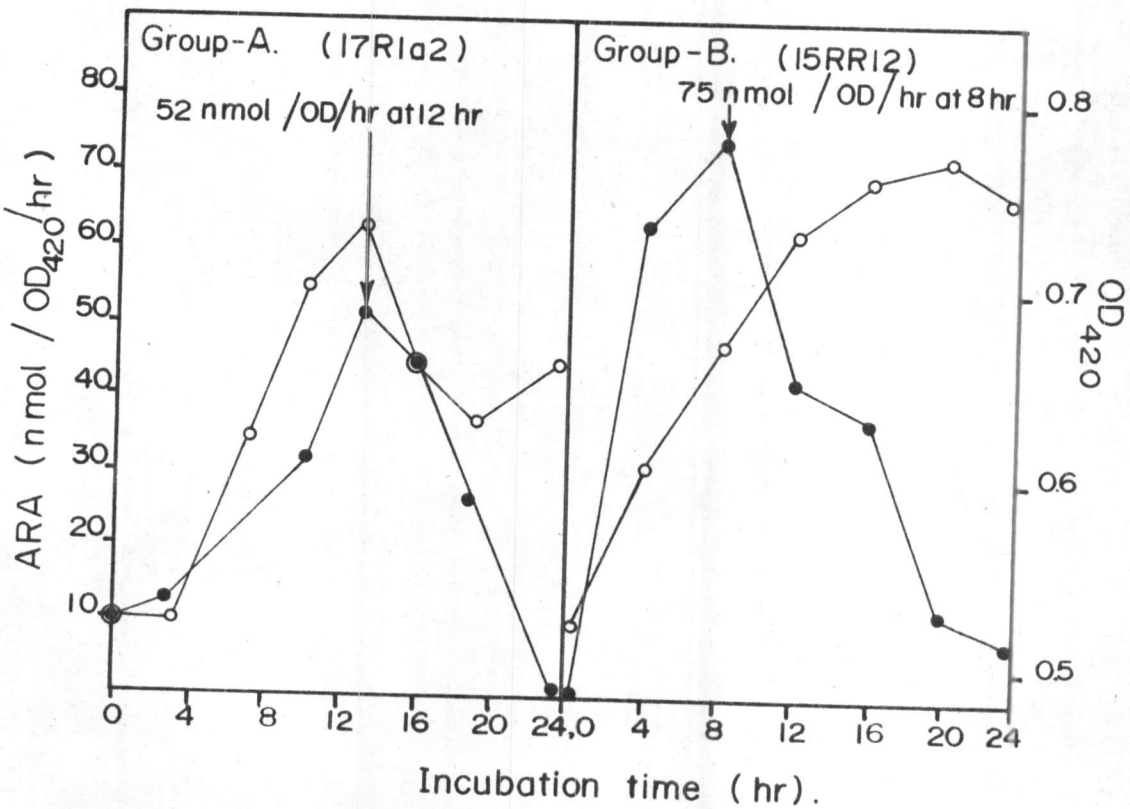
Figure 13. The relationship between ARA and stage of growth of the rhizospheric diazotrophs.

The bacterial cells harvested in the midlog phase were suspended in NF medium. Each culture was divided into 14 identical tubes with the starting concentration of 0.52 OD_{420}. The first two tubes were assayed for ARA at 0 time. The rest were incubated at room temperature (29-30°C). At various time intervals, a duplicate of samples were analyzed for ARA and OD_{420}.

●——● ARA

O——O Optical density at 420 nm.

Each point represents the mean of 2 samples.


Group-A. (17R1a2)
52 nmol /OD/hr at12 hr
Group-B. (15RR12)
75 nmol / OD/ hr at 8 hr
ARA (n mol / OD420/hr)
OD420
Incubation time (hr).
0
4
8
12
16
20
24,0
24
10
20
30
40
50
60
70
80
0.5
0.6
0.7
0.8