CHAPTER IV.

DISCUSSION.

1. The sample collection.

To approach the aim of increasing the N_2-fixing potential in the North-Eastern part of Thailand, the three rice experimental stations were chosen for collecting rice root and rhizospheric soil samples. The two rice experimental stations, Tapra and Chumpae, are in the North-Eastern part and the other, Rangsit is in the central plain. The soil type of all the three sites are not perfect for rice cultivation. At Tapra the soil is sandy (85% sand in soil composition) and the pH is nearly neutral (6.1). At Chumpae, the soil pH is more acidic than Tapra (5.8) but the sand per cent in soil is lower (57%). at Rangsit, the clay per cent is high (60%), so the water holding capacity is better than the soil at Tapra and Chumpae, but the soil pH was quite acidic (pH 4.0). The N_2-fixing potential associated with the rice root at the three sites according to soil type, fertilizer application and the rice varieties were compared. In 1978, Wada *et al.*,(44) measured the N_2-fixing potential associated with flooded water, floating weed and the rice rhizosphere *in vitro* by C_2H_2-reduction method as influence by the different fertilizer treatment, non-fertilized plot (NF), inorganic fertilizer (IF), green manure (GM) and organic manure (OM). The N_2-fixing activity of all samples at different fertilized plots showed the same profiles. Difference in the N_2-fixing activity among the 4 plots

were most prominent at the maximum tillering stage. The four plots were arranged in the following order according to the increasing N_2-fixing activity : NF < IF < GM < OM. Similar effect of the fertilizer application on the N_2-fixing activity was also found by Panichsakpatana, et al.,(45). We also considered this factor. At Rangsit, we compared the influence of organic matter (rice straw compost in T_5), and chemical fertilizer (T_6) on the N_2-fixing potential. At Chumpae, PK-fertilizer and NPK-fertilizer application were compared. Balandreau, et al.,(46) studied the effect of plant species on N_2-fixing activity. They compared the ARA associated with peanut, rice and grass. Dobereiner, et al.,(26) and Eskew et al., (47) studied the effect of the varieties of grass on N_2-fixation. In the case of rice varieties, Hirota,et al.,(27) and Watanabe, et al.,(48) reported the different ARA values in different rice varieties. They suggested that the rice plant genotype influence the association between the N_2-fixing bacteria and rhizosphere. We concerned this effect in this research at Tapra, therefore the sticky rice varieties (Sanpatong) and RD.6 were compared with the non-sticky rice (Kowmali and RD.15) varieties.

The rice roots and rhizospheric soil samples were collected from the three experimental sites, Tapra, Chumpae and Rangsit, throughout one rice growing season. We should follow the ARA of every stage of rice growth, because of the fluctuation of the ARA in different stages of their growing season. Dommergues, et al., (49) reported the daily variation of ARA even in only one stage of rice growth.

2. The sample preparation.

The collected rice roots were washed by tap water until free of soil to remove the microorganisms habitated in the soil particle adhered to the root. Panichsakpatana, et al.,(45) compared the N_2 -fixing activity of the unwashed and washed roots in Japan they reported that most of the active N_2-fixing microorganisms inhabited on or inside the root at the early stage, as the N_2-fixing activity of the washed root was similar to the unwashed root. On the contrary, the N_2-fixing activity of the washed root was far lower than that of the unwashed root at the later stage because the soil particles which adhered to the roots were the main micro-habitate for the N_2-fixing microorganisms. The same results were obtained by Watanabe, et al.,(48). However Dobereiner, et al.,(26) studied bacteria associated with the grass root, Paspalum notatum by cutting the roots into thin sections, they found the mucigel layer which was stained metachromate red with taluidene blue. This result indicated that the colonies of bacteria attached on the root surface associated with the mucigel layer which might provide the right condition for fixing nitrogen and also protect the cells from being washed of from the roots. Thus the rhizoplane bacteria of the rice root should remain on the washed roots by similar association.

The roots free from the soil were the subject to study the ARA of rhizoplane and endorhizospheric bacteria. Some of the rice roots were surface sterile by chlorox and ethanol. The purpose was to eliminate the rhizoplane bacteria associated with the root

surface and examined only the N_2-fixing activity of the endorhizospheric bacteria.. Watanabe, et al.,(48,50) suggested the other method. He seperated the rhizoplane and endorhizospheric bacteria from the washed roots by shaking the roots vigorously with glass beads to remove rhizoplane bacteria and then the roots were blended to provide a suspension of inner rhizoplane bacteria. He did not measure the N_2-fixing activity of the microorganisms in the associated condition with the rice root, which was the disadvantage of this method. In this study, the washed roots and the surface sterile roots were cut into segment about 1-2 cm long and randomly weighed out 0.5 gm for each sample. Panichsakpatana, et al.,(45) reported that the N_2-fixing activity cannot be expected to be uniform throughout the whole root system of one rice plant. This effect was clearly understood in unwashed root, because the type of N_2-fixing bacteria attached were different symbiotic and saprophytic species. His experiment in Japan indicated that, at the early stage, the symbiotic species was dominant, whereas the saprophytic species was dominant at the later stage. Although, his data showed only slightly effect in the case of washed root, we have considered this factor and therefore the roots were cut and randomly sampled.

The rice roots and the rhizospheric soil samples were preincubated in NF or NF supplymented with organic N (YE). This was done because Watanabe,et al.,(48,50), reported the enhancement of ARA by the supplyment with YE. The samples were then preincubated in these media about 40 hours.

Since Simasatitkul, et al., (51) and Neyra, et al., (14) reported that excised rice and grass root assayed for ARA showed a lag period of 6, and 8-18 hours before ARA could be observed and the preincubation for 40 hours, can solve this problem. However the proliferation of the bacteria also occured during preincubation, therefore the ARA after this preincubation was according to the proliferating number of nitrogen fixing bacteria.

3. ARA associated with the rice roots and rhizospheric soil.

The biological nitrogen fixation in the rhizosphere of rice plant in this research was measured by acetylene reduction method in the laboratory condition. By using this method, it was realized that, while the acetylene was reduced by nitrogenase enzyme, nitrogen gas in the assayed flask was not reduced because acetylene is the noncompetitive inhibitor of nitrogen (52) and ethylene produced was not further reduced to methane (45).

When C_2H_2 was injected into the assayed flask as the substrate for studying the nitrogenase activity, some experiments showed the lag period of the enzyme activity. Simasatitkul, (51) measured ARA associated with rice rhizosphere, in vitro. After the samples were prepared in the assayed flask, they were not preincubated, but the air was suddenly replaced by 0.2 atm C_2H_2. The C_2H_4 production was detected immediately and furthered on until 48 hours of incubation. The result showed that the lag period was within 6 hours of incubation. Hirota, et al., (27) measured ARA

associated with excised root, whole plant with soil kept in a polyethylene bag, and soil alone. The samples were in the gas mixture of 0.1 atm C_2H_2 and 0.9 atm He. The ARA of all the samples showed the lag period of 6 hours which corresponding with Simasatitkul. In addition, Hirota, et al.,(27) showed that an intact plant with undisturbed root system enclosed in a vessel such as a descicator to minimize the exposure of the rhizoplane to air, in this case only the production of C_2H_4 from C_2H_2 was linear and no lag period of the reaction was observed. This conclusion was obtained in different gas phases; 0.1 atm. C_2H_2 and 0.9 atm. He or 0.1 atm. C_2H_2 and 0.9 atm. air. Thus, the lag period of the activity depended upon the exposure of the root to the air before the C_2H_2 incubation. To minimize the lag period, we therefore preincubated our sample for 40 hours before C_2H_2 incubation.

Yoshida, et al.,(35) reported that several crops produced C_2H_4 which caused the overestimation of the ARA. Simasatitkul,(51) examined the C_2H_4 produced by rice roots in the condition that resembled ARA determination, but failed to detect any C_2H_4 production by rice root. In addition, he examined the consumption of C_2H_4 by rice root, he found a small decrease of C_2H_4 after incubation, which might result from dissolving of C_2H_4 in the medium or leaking of C_2H_4 from the assayed flask.

In this experiment two "blank" flasks were set for each set of samples, one contained sample in the medium without C_2H_2 injection and the other contained only medium and C_2H_2 in the flask, but no

sample. Rarely we found C_2H_4 production in some of the former blank flasks which would be subtracted from that set of samples. This C_2H_4 could be produced by the rice root or associated microorganisms.

4. The N_2-fixing potential profiles.

Soil properties was the important factor, that affected N_2-fixing potential profiles. This was evidenced by Figure 4 in which the N_2-fixing potential profiles of the same rice variety (RD.7) at Chumpae and Rangsit in the nonfertilized plots were compared. Different N_2-fixing potential profiles were observed in different soil type.

In 1973, Dommergues, et al.,(49) studied the ARA of Eleusine coracana (a maize variety) in different soil type under laboratory condition. He used the 1 month old plant in the soil and plant system and found that the activity associated with the plants grown in soil, pH 6.0 was 100-fold higher than in soil pH 4.5. This result supported our results, because the N_2-fixing potential at Chumpae, soil pH 5.8 was significantly higher than at Rangsit, soil pH.4.0

The soil type might affect the association between the diazotrophs and roots, at Rangsit the rhizoplane plus endorhizospheric N_2-fixing potential (A) was almost the same as the endorhizospheric potential (B), indicating that most of the N_2-fixing bacteria inhabited inside the root.

At Chumpae both types of association, the rhizoplane and endorhizospheric N_2-fixing potential were observed. The results also

suggested that there could be a transition of the bacterial population from the rhizoplane towards the inside of the root in the early reproductive phase. On the contrary, Panichsakpatana, et al., (45) reported that in the early stage the bacteria attached firmly to the rice roots, and seemed to fall off in the later stage possibly because of root degeneration.

In conclusion the dominant N_2-fixing potential at Rangsit was in the inner rhizosphere because of the acidic soil (pH 4.0) with the maximum in the ripening stage. At Chumpae, (pH 5.8, slightly acid) the N_2-fixing potential resided in both inner rhizosphere and rhizoplane with the maximum in the tillering stage. Thus, the soil type may influence the N_2-fixing potential profile via the domination of habitation of the N_2-fixing bacteria.

The influence of different fertilizer application on the N_2-fixing potential profiles, were shown in Figure 5 and 6, in which the fertilized plots and the non-fertilized plots were compared. It can be concluded that the fertilizer application did not significantly change the N_2-fixing potential profiles at Rangsit, possibly because most of the diazotroph inhabited in endorhizosphere. Application of rice straw compost in T_5 and T_{10} was noticed to decrease the N_2-fixing potential. One explanation could be that the decomposition of the rice straw released toxic substance (38) and organic acids (53) which should increase the acidity of the soil. Since Rangsit soil is already acid, pH 4.0. This might inhibit the growth of free living, and the rhizoplane bacteria, therefore

reducing the N_2-fixing potential og the Rhizoplane. Secondly according to Prabuddham (41), the content of nitrogen in rice straw was about 0.6 % therefore the total organic N content in the 12 ton rice straw was approximately 70 kg N. Based on this estimation, the total N-fertilizer applied in T_{10} was about 120 kg N. Altogether, Rajaramamohan Rao, et al., (53) used the combination of $(NH_4)_2SO_4$ and rice straw application, and measured the N_2-fixing activity by both ^{15}N and acetylene reduction method. He reported that combined N when applied at 65 ppm which corresponded to the rate of 100-150 kgN/ha partially inhibit N_2-fixing activity.

This inhibitory effect of N-fertilizer (100-150 kgN/ha) was also observed in the endorhizospheric activity of T_5 in Chumpae.

When the N_2-fixing potential profiles at Tapra, with high rate of chemical fertilizer application (NPK = 136,27,5 kg/ha) were compared with T_6 at Rangsit (50,25,25 kg/ha) and T_5 at Chumpae (113,38,25 kg/ha). It is obvious that the N_2-fixing potential profile at Rangsit and Tapra showed the maximum at different phase of growth, the former in reproductive phase, and the latter in vegetative phase Comparing between Tapra and Chumpae, again the difference in the profiles from the two sites can be observed. Although the maximum potential was common in the tillering stage, but at Chumpae the high potential in the profiled prolonged throughout the rice growing season, whereas the potential at Tapra decreased to lower levels in the later stage of growth.

Thus, the three rice experimental sites with N,P,K,

-fertilizer

application performed differing N_2-fixing profiles.

The influence of the rice varieties, non-sticky rice, Kowmali, (the old rice variety) and RD. 15 (the new rice variety), and sticky rice Sanpatong (the old rice variety) and RD.6 (the new rice variety) on the N_2-fixing potential profiles, was shown in Figure 7. The rhizoplane and endorhizospheric N_2-fixing potential (A) associated with sticky and non-sticky rice showed similar profiles. The high N_2-fixing potential of every variety was in the tillering stage as shown in Figure 7. However, the old variety, Kowmali seemed to show higher rhizospheric N_2-fixing potential than the new variety, RD. 15. But for the 2 sticky rices the old variety, Sanpatong and the new variety, RD. 6 showed the same level of N_2-fixing potential. The application of YE seemed to enhanced the potential associated with the two sticky rice varieties. Thus the difference in the four rice varieties, we have studied, did not change the N_2-fixing profiles and slightly affected the maximum potential.

In conclusion, among soil types, fertilizer application and rice varieties, we concluded that the soil type is the most affected the N_2-fixing potential profile, whereas the other two factors mainly affected the degree of maximum activity in each stage.

When the amount of nitrogen fixing potential at each stage was summed up on basis of area under peaks, the total nitrogen fixing potential per crop demonstrated in Figure 8 indicated that the basal nitrogen fixing potential in the three rice paddies was in the range 19-72 kgN/ha/crop. Supplementation of NF medium with yeast extract

promoted the total nitrogen fixing potential to the range of 30-89 kgN/ha/crop. The amount of total nitrogen fixing potential per hectare per crop in both conditions, although they were high estimation because of the bacterial proliferation during preincubation were the important basic information indicating the original N_2-fixing potential already existed in the paddy fields of Thailand. This information is essential for the evaluation of the future promotion of the biological nitrogen fixation in these areas, such as by the application of the selected rhizospheric nitrogen fixing bacteria back to the field.

5. The morphology and ARA of the isolated N_2-fixing bacteria.

The colonial morphology and cell structure of the bacterial culture isolated from Chumpae, Rangsit and Tapra grown on NF-plate were more or less similar. The colonies formed were slimy, circular shape, with pin-point size. They could not produce pigment. All of them were Gram negative bacteria.

The difference in the number of incubation days for colony formation, however indicated the difference in homogeneity of the bacterial population among the three sites. The order of degree of homogeneity was Chumpae > Rangsit > Tapra. The bacterial cultures isolated from Chumpae were formost the one day fast growing bacteria, whereas the bacteria from Rangsit distributed themselves half and half between 1 and 2 days of incubation. The bacteria isolated from Tapra showed very wide distribution of incubation days (1-5 days).

In addition, the bacteria isdlated from the new rice variety, RD.15 and RD.6 seemed to be consisted of more homogeneous population (one day of incubation) than the old varieties (one and two days of incubation.)

The histrograms of the number of bacteria and the different levels of ARA shown in Figure 9 also evidenced for the difference in bacterial population at Tapra from Chumpae and Rangsit. We found that more than half of the cultures isolated from Tapra possessed relatively high ARA. At Chumpae and Rangsit, the diazotrophic population seemed to distribute evenly among the four classes of ARA.

All the cellular morphology of these nitrogen fixers were examined in stationary phase, therefore pleomorphic growth could not be observed. However, Eskew, et al.,(47) reported the pleomorphic growth of Azospirillum lipoferum (Spirillum lipoferum) isolated from a kind of coastal grass which showed the characteristic spiral shape and motility in younger culture and developed larger, nonmotile, cystlike form in older cultures.

6. The characteristics of the selected rhizospheric diazotrophs.

The eight selected diazotrophs showing high ARA were classified into three catagories.

Group A. The optimum temperature was 37°C when grown in NF + 10 % rich medium, they preferred slightly basic and neutral pH The maximum ARA was at the early stationary phase (52 nmol/$_{420}$/hr.)

Group B. The optimum temperature was also 37°C when grown in

NF + 10 % rich medium. They were more sensitive to slightly acidic pH than Group A. However, the maximum ARA resided in the midlog phase was higher than Group A. (75 nmol/OD_{420}/hr.)

Gruop C. They were the thermophillic bacteria with the optimum temperature in the range at 42-46°C when grown in NF + 10 % rich medium. The special property was the changing in pH (5.5-7.5) could not affect their growth. Although its maximum growth was low (25 %) compared with those in Group A and B.

From these characteristics, Group C should be able to survive in acid soil such as Rangsit, (soil pH 4.0). In addition Group C preferred high temperature, they should be suitable for the high temperature in the rice field at the North-Eastern part.

In the last experiment, we did not succeed in finding the relationship between the ARA and the growth of bacteria in Group C. It might be, because of the lack of some necessary factors required for the pure bacteria to express the N_2-fixing activity after being isolated from the rhizospheric ecosystem for a period of time. Our opinion, came from the previous results that these selected strains (10Sa21, 25Sa12) were able to show high ARA (more than umol/OD_{420}/d). Several transfers of these bacteria from associative state with the root to the pure cultures may cause dilution and finally deficiency of some important factors. Because we also found that, the culture isolated from high ARA root pieces and rhizospheric soil samples might not show corresponding high ARA as the original samples, Eskew, et al.,(47) reported similar phenomena

in the isolation of pigmented Azospirillum lipoferum from the roots of Cynodon dactylon. He found that the ARA of the root pieces and the derived cultures showed differing levels of activity.

In an analogous system between legume and Rhizobium sp., it has been shown by Pagan, et al.,(54) that the legume metabolices were secreted and were the inducer of nitrogenase activity of the Rhizobium spp. They reported that pure culture of Rhizobium spp. in the absence of host plant, did not show nitrogenase activity unless arabinose, succinate or glutamate was added into medium. In our case, we have tried arabinose, succinate and glutamate supplementation for the bacteria in Group C, but the C_2H_2-reduction activity detected was still very low or undetectable. Therefore at present we did not find " the missing factor (S) " necessary for the rejuvenation of growth and the nitrogenase activity of the bacteria in Group C.

By comparing the list of aerobic heterotrophic N_2-fixing bacteria reviewed by Burns and Hardy (6) and their characteristic (55), our three groups of isolated bacteria showed the characteristics which fit the descriptions of Derxia gumosa and the currently defined Genus, Azospirillum spp. (14,56,57,58) which consisted of two species A. lipoferum and A. brasilense as summarized in Table 13. All of them are the root associated nitrogen fixer in the Tropic.

The bacteria in Group A and B resemble to Derxia gumosa by showing the same range of optimum temperature 35-37°C, production of slime, and acidification of glucose medium.

Table 13. The taxonomic characteristics of some aerobic diazotrophic bacteria.

Comparison of the three established aerobic diazotrophic bacteria: Derxia gumosa, Azospirillum lipoferum, Azospirillum brasilense and the aerobic diazotrophic bacteria isolated from the rice rhizosphere grown at Chumpae, Tapra and Rangsit.

(NT - not tested)

* (58)

** (14,56,57)

	Aerobic diazotrophic bacteria						
	Group A	Group B	Group C		Derxia * gumosa	Azospirillum ** lipoferum	Azospirillum ** brasilense
			10 Sa21	25 Sa 12			
Optimum temperature (°C)	37	37	46	42-46	35 - 37	35	35
Slime	+	+	+	–	+ +	–	–
Pigment in rich medium	pale yellow	pale yellow	pale yellow	pale yellow	± (+ with age)		
in N-deficient medium	–	–	–	white	± (+ with age)		
Cell shape	small rod	small rod	small rod	large rod	small rod	small curved	small curved
Biotin requirement	–	–	–	–	–	+	–
Glucose as sole carbon in N-deficient medium	+	+	+	+	+	+	–
Acidification of glucose-yeast extract broth	+	+	+	+	+	+	–
Growth in glutamate medium	+	+	NT	NT	+	+	+
Catalase	NT	NT	NT	NT	–	+	+
Sole carbon source ribose			–	–		+	–
xylose			+	+	–	+	+
arabinose			+	–		+	+
glycerol			+	+	+	+	+
lactate			+	–	–	+	+
succinate			+	+	–	+	+
mannitol			+	–	+	+	–
citrate			–	+	–	+	+

The bacteria in Group C differs from Derxia gumosa in their utilization of sole carbon source in nitrogen deficient medium. They resemble Azospirillum sp. in their ability to use lactate, succinate, citrate and xylose as carbon sources.

From the taxonomic characteristics of Azospirillum lipoferum, Azospirillum brasilense and other aerobic diazotrophs it can be seen that further characterisation of our isolated bacteria should be conducted in the following aspected;

1. The G + C content of DNA, both Derxia sp. and Azospirillum sp. contain about 70 % of G + C content which differ from those of Azotobacter sp. (65 %)

2. Testing for catalase activity, which can distinguish Derxia sp. from other species.

3. Testing for the ability to use various carbon sources such as ethanol, sorbitol, pyruvate, galactose and sucrose.

4. Changes in cell morphology in different media and phases of growth.