CHAPTER I.

INTRODUCTION.

1. The Nitrogen Cycle

Nitrogen is a key building element of protein and nucleic acid molecules upon which all life is based on, thus it is an indispensable component of simple organisms such as bacteria as well as complex organisms like higher plants and animals. The nitrogen present in animals are obtained from food which are directly or indirectly derived from plant tissues (Figure 1) therefore increasing crop yield certainly increases nutritional materials for animals (1). All of vegetational cover of the earth is dependent on inorganic nitrogen for growth, so it requires continual conservation and maintenance of this form of nitrogen. The available form of nitrogen for plants are ammonium-nitrogen and nitrate-nitrogen. Plant gains the available nitrogen either from man-application of nitrate, ammonium and urea fertilizers, or from several natural processes. The first and major natural process is the mineralization of soil organic nitrogen into available form by soil microbes (2). Secondly the fixation of dinitrogen (N_2) in the atmosphere into nitrate and nitrite via spontaneous reaction with oxygen during the thunderstorm, and into ammonia via a biological pathway.

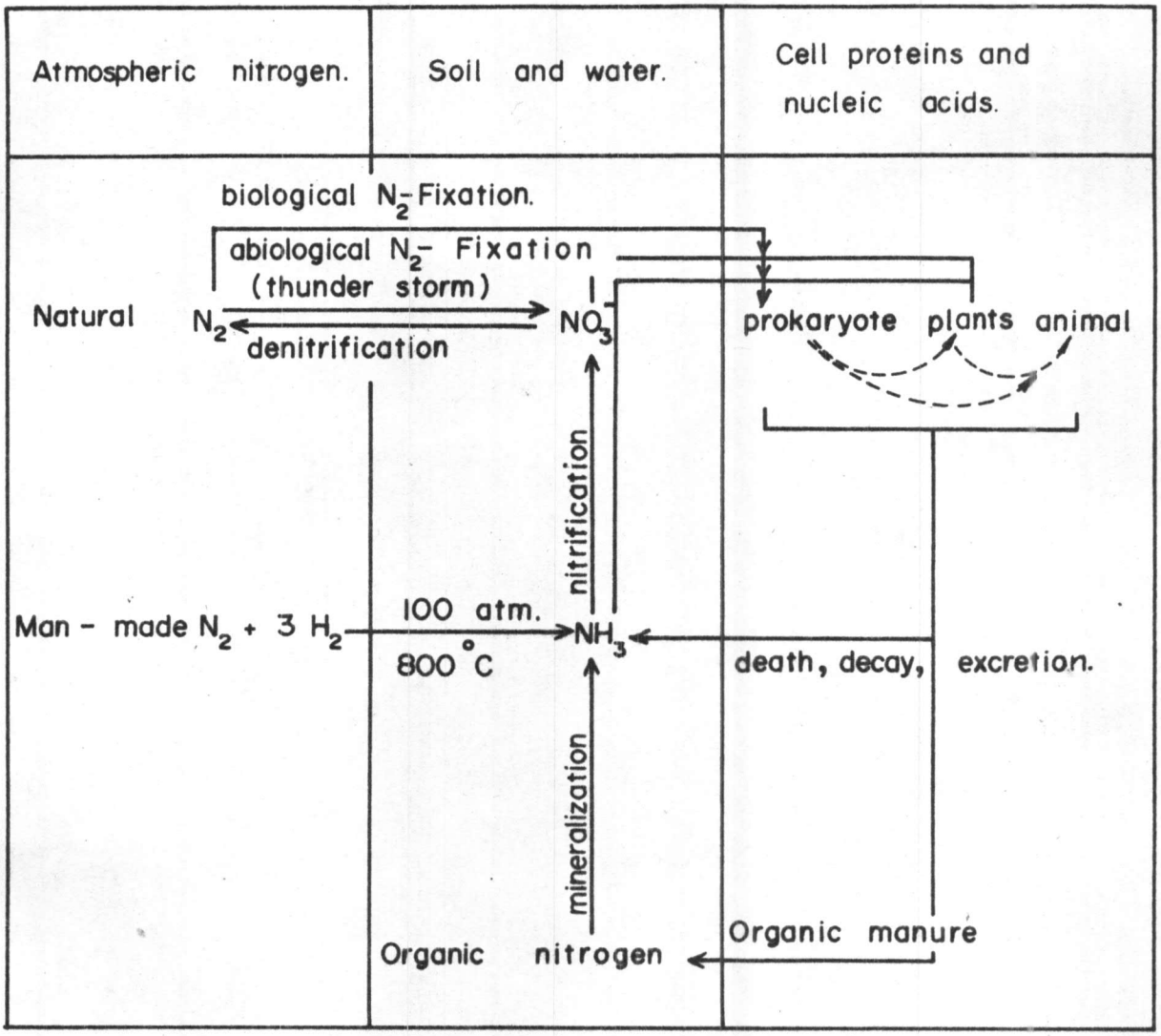

Figure I. The principle reactions of the nitrogen cycle, modified from Sprent, 1979. (3)

2. The dinitrogen fixation

Fixation of molecular dinitrogen, man-made or natural into other compounds constitutes the primary input of nitrogen into the biosphere (4).

2.1 Man - made industrial process.

Nitrogen and hydrogen can be combined by the industrial Haber-Bosch process to form ammonia which can be used for fertilizer at the expense of the fossil fuel. It was estimated that about 1.5 kg. of fuel oil is needed to manufacture and deliver 1 kg. of fertilizer to the farm (3). According to the 1975 UN statistics 48 TgN (TgN = 10^{12} gram nitrogen) was fixed by the world production of nitrogen fertilizer in 1975 (4).

$$N_2 + 3H_2 \xrightarrow[800^{\circ}C]{100\ atm} 2NH_3 \quad \ldots\ldots\ldots (1)$$

2.2 Natural nitrogen fixation process.

2.2.1 Fixation by lightning.

A review on fixation by lightning by Soderlund and Svensson (5) gave the idea that the atmospheric nitrogen is fixed, by the oxidation of electric discharge from the lightning phenomenon, to the nitrite or nitrate form, but the amount of atmospheric nitrogen fixed by this process was concluded to be of minor importance for the nitrate content of rain water. Burns and Hardy (6) also reported the amount of N_2 fixed via oxidation by lightning is about 20 % of nitrogen precipitated as NO_2^- / NO_2^- .

2.2.2 Biological dinitrogen fixation.

In the biological system, nitrogen-fixing organisms light energy, directly or indirectly to produce.

ammonia from atmospheric nitrogen and since manufacture occures on the site, delivery costs are not necessary. This energy-saving potential of biological nitrogen fixation is therefore ranked its importance.

2.2.2.1 The three types of biological N_2-fixation.

All the organisms that can utilize atmospheric nitrogen (78% of the air) belong to the kingdom known as the prokaryotes. The three types of biological N_2-flxation are as follow (6).

a. Obligatory symbiotic type.

The symbioses bring together two species, neither of which can fix N_2 independently. The inability of either partner to fix N_2 independently is the qualifying characteristic which distinquishes these obligatory symbioses from the other two types. The Phizobium - legume root nudule symbioses are the only definitive examples of obligatory symbiosis (3,7).

b. Associative symbiotic type.

A great variety of associations exist between free-living nitrogen-fixers (diazotrophs) and other organisms (non-diagotroph). Most of these association are based on the non-diazotroph contributing carbon compounds to the diazotroph, usually via an exudate, and the diazotroph contributing nitrogen to its host, usually in the form of N - containing compounds generated in the decomposition of dead diazotroph cells, i.e. organisms capable of decomposing polysaccharides have been observed to stimulate N_2 fixation such as Anabaena sp. and Azolla sp. association (8,9),

Panicum maximum associated with Spirillum lipoferum (10,11), Azotobacter chroococcum, A. vinelandi, Azospirillum sp. associated with maize roots (12,13), Beijerinckia, sp., Enterobacter, sp. Azospirillum sp. associated with rice roots (12,14,15).

c. Free living type.

The free living diazotrophic microorganisms are independent microorganisms. Three catagories of them are classified according to their demand of oxygen.

(a) Aerobic diazotrophs, which have evolved several ways of living in an aerobic environment are Azotobacter sp., Derxia sp., (13) and some blue green algae, such as Anabaena sp., Glocotrichia sp., and Nostoc sp. (16).

(b) Facultative diazotrophs habitate in the aerobic, micro-aerophillic and anaerobic environment such as Klebsiella sp., (17) and some photosynthetic bacteria, i.e. Rhodospirillum sp., (18)

(c) Anaerobic diazotrophs appear in the anaerobic environment such as Clostridium sp. (19).

The basic nitrogen fixation reaction of all the diazotrophic microorganisms appears to be the same in all cases, i.e. the reduction of N_2 to NH_3 with the aid of the enzyme complex named nitrogenase.

2.2.2.2 The Nitrogenase (20)

The nitrogenase system can be seperated into two components. One of these, azoferredoxin (azoFd) is an extremely oxygen sensitive iron-sulfer protein. Azoferredoxin consists of two identical peptide chains of M.W. about 30,000. Each dimer contains 4 iron atoms, $4S^{-2}$ and 12 tetratable thiol groups. The other component molybdoferredoxin (MoFd) contains both iron and molybdenum as well as labile sulfide. This protein contains two kinds of peptide chains of the M.W. 51,000 and 60,000 in a mixed ($\alpha_2\beta_2$) tetramer. Each mixed tetramer contains two molybdenum atoms, approximately 24 iron atoms, 24 S^{-2} and 30 titratable thiol groups, possibly in the form of three Fe_4S_4 clusters. The protein associate in a ratio of two dimeric azoFd molecules to one MoFd to form the nitrogenase complex.

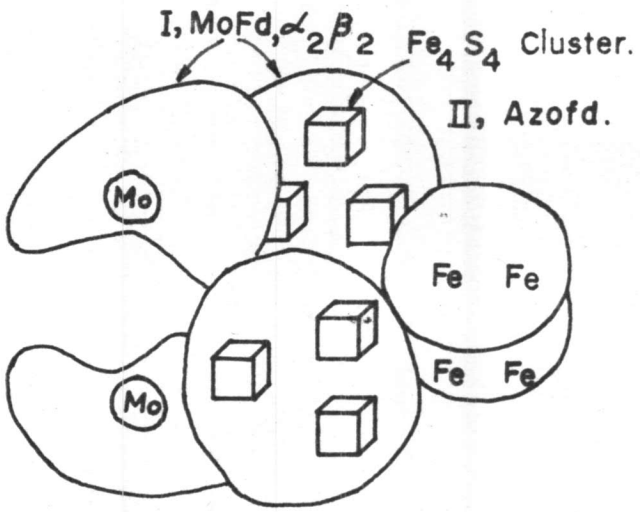

Figure 2 Hypothetical structure of a nitrogenase molecule, from Metzler, 1977 (20).

2.2.2.3 Dectection of biological nitrogen fixation.

As research in N_2 fixation and analytical technique advanced, a variety of assay methods for N_2 fixation were developed, the observation of growth and morphology of legume root nodule development is one of the most simple indirect methods. Analysis of increasing N_2 content after N_2 fixation in a specific condition is also possible by using the Kjeldahl method (21). However, to achieve higher sensitivity another two techniques are widely used:

a. $^{15}N_2$ method

The application of the stable isotope ^{15}N; by the preparation of N_2 enriched in ^{15}N and measure the $^{14}N/^{15}N$ ratio in a masspectrometer. Burns and Hardy, (6) reported the use of this technique to study N-asimilation in Azotobacter provided a sensitivity about 1000 - fold greater than that of the Kjeldahl's method.

b. Acetylene reduction method.

The discovery that nitrogenase could reduce compounds other than N_2 led to the development of acetylene reduction assay for N_2 fixation. Dilworth, (22) discovered that nitrogenase could reduce acetylene (C_2H_2) to ethylene (C_2H_4). The analysis were conducted by incubating the test material in a gas-tight container which contains a partial pressure of C_2H_2. At an appropriate interval, samples of the atmosphere was withdrawn by a gas tight syringe for immediated analysis of C_2H_4 production in a gas chromatograph. The reduction of 3 moles of C_2H_2 to C_2H_4

was equivalent to the reduction of one mole of N_2 as shown in equaion (2) and (3) . The factor for estimation N_2 fixation from C_2H_2 reduction is called conversion factor, which was theoretically 3. The variation of this factor was discussed by Hardy snd Burns (23) .

$$N \equiv N + nATP + 6e^- \xrightarrow[\text{nitrogenase}]{} 2NH_3 + nADP + nPi \quad \ldots\ldots(2)$$

$$HC \equiv CH + nATP + 2e^- \xrightarrow[\text{nitrogenase}]{} H_2C{=}CH_2 + nADP + nPi \quad \ldots\ldots(3)$$

The sensitivity of this method is claimed to be about 10^3 times greater than the $^{15}N_2$ method (6)

2.2.2.4 Evaluation of biological N_2 fixation associated with the rice plant by acetylene reduction method.

There are two types of measurement by the acetylene reduction method.

a. In Situ method, acetylene reduction method involves the intact plant system in the pot or in the field. The assay is performed by using a plastic bag covering each rice hill in the rice paddy, connecting with the cylindrical base to seperate the rhizospheric part from its surrounding. Acetylene was applied to this closed system, and atmospheric sample was drawn later on and measured in a gas chromatograph. This method is based on two assumtions. First, C_2H_2 must reach the N_2 fixing site, and the other, the C_2H_4 that evolves from C_2H_2 reduction at the N_2 fixing site must be quantitatively recovered from the assay chamber. Lee, et al. (24) reported that stirring the water-saturated soil before sampling reduces the amount of C_2H_4 remaining in soil. Diffusion of C_2H_2 and C_2H_4 through water saturated paddy soil by themselves are very slow,

but the rice plant has a well-developed gas transporting system to carry o_2 and N_2 from the aerial parts through out the basal parts of the roots. It was assumed that the C_2H_2 supplied to the N_2 fixing sites near or in the rice roots is more likely dependent upon the gas transporting system rather than upon simple diffusion. It is still doubtful whether this *in situ* field assay techniques could detect nitrogen-fixation in anaerobic soil remote from roots. In 1979 Watanabe, *et al.*, (25) has modified this technique by using the intact rice plants grown in water culture, and measured their associated ARA in closed assay chambers to eliminate the restriction of slow transfer of C_2H_2 and low recovery of C_2H_4 in a water saturated soil system.

b. *In vitro* method.

Although this method did not involve ecosystem of the rice plant like the first method, but some advantages of this method should be considered. We could compare for the more important N_2 fixing sites among water, remote soil from roots, rhizospheric soil, excised rice root (25,26,27) and surfaced steriled root. The ARA associated with the inner and outer rhizosphere could also be compared (28). The samples were preincubated in the media in assayed flask for a period of time and then C_2H_2 was administered, and C_2H_4 produced was measured. The disadvantages of this method are the followings. Firstly, the exposure of the rhizosphere to the air reduce C_2H_2 reduction activity (ARA) considerably, because of damage of nitrogenase by oxygen (26,27).

In addition, the overestimation of ARA resulted from the proliferation of microorganism during preincubation before C_2H_2 reduction assay in the media should be considered (27).

3. Nitrogen Fixation in lowland rice paddy ecosystem

It has long been observed that a mechanism for maintaining nitrogen fertility should be operated in a submerged rice ecosystem. For example , Okuda, (29) showed that the rice yield in a flooded plot without nitrogen fertilizer stayed almost constant for several years. In Japan and in Southeast Asia, rice has been grown for many centuries without chemical nitrogen fertilizer and in some places, a considerable rice yield has been continuously obtained without adding nitrogen fertilizer. Therefore, it is reasonable to assume that nitrogen input took place by some means to compensate for the removal of nitrogen by the crop. Biological N_2 fixation may partly explain this long term nitrogen fertility in a submerged soil ecosystem. The principal agents of biological N_2 fixation in a rice paddies are as follow (30,31) , and is shown in Figure 3.

1. Free living blue green algae

2. Associative symbioses between blue green algal and the water fern (Azolla)

3. Heterotrophic bacteria

3.1 in the rice rhizosphere

3.2 in the soil remote from the rice root.

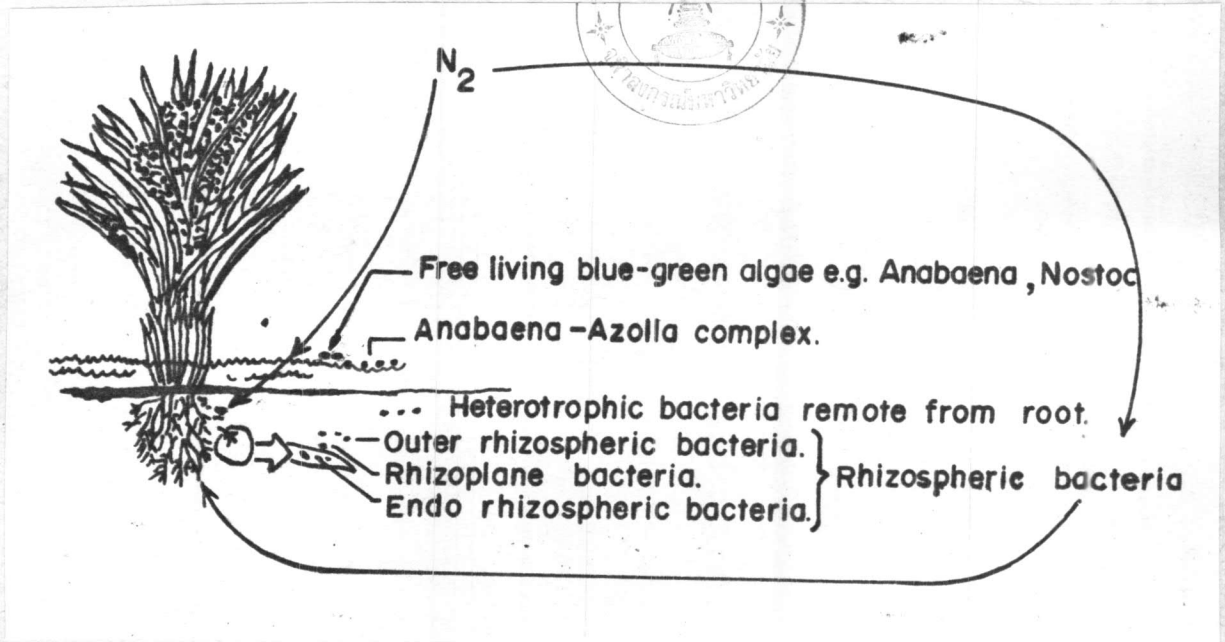

Figure 3 The principal agents of biological N_2 fixation in a rice paddies

Although high potential of N_2 fixation was reported in the blue green algal-azolla complex (N_2 fixing rate 450 kg N/ha/hr)(30). It requires permanent supply of irrigated water and direct light energy. On the other hand heterotrophic nitrogen fixing bacteria can survive in merely rained fed paddies and use chemical energy.from organic matters in the soil. Moreover the transport of N_2-fixed by the rhizospheric diazotroph into the associated rice root should be more efficient than from the azolla system. Thus, the role of rhizospheric N_2 fixing bacteria is considered as the most promising system in the nonirrigated rice area of Thailand.

Associative heterotrophic diazotroph in the rice rhizosphere.

Mac Rae and Castro (31) reported that rice root excreted some carbohydrates and amine acids. These root exudates attracted the heterotrophic bacteria to the rice rhizosphere, so that the population of the bacteria attached to root surface is greater than the sites remote from the rice roots. These heterotrophic bacteria are mostly N_2-fixer, thus the rice plants can gain N_2-fixed by these bacteria.

The rhizospheric diazotrophic bacteria associated with the rice root can be devided into three catagories (32).

1. Outer rhizospheric bacteria is the bacteria which inhabits in the soil adhering roots, so if we washed off the soil from the root by tap water, these bacteria should be removed out.

2. Rhizoplane bacteria is the bacteria which associate firmly with the root surface, and remained attached to the root surface after washing with tap water.

3. Endorhizospheric bacteria is the bacteria which penetrate into the cortical layer of the root, and thus be protected from surface sterilization.

4. The research on rhizospheric biological nitrogen fixation in the rice paddies.

In 1959, Chakarborty and Sen Gupta (33) cultivated the Oryza sativa, variety Amen, in liquid nitrogen-free (NF) medium and found a gain of 50 percent of the nitrogen content in 7-week old

plants. They suggested that irce plants might be capable of fixing atmospheric nitrogen. In 1971 , Yoshida and Ancajas (34) carried out similar experiment, but using steriled rice seed grown in steriled flooded soil, they could not detect nitrogen fixation. They therefore concluded that nitrogen was fixed by nitrogen-fixing bacteria associated with the rhizosphere. Later in 1973, Yoshida and Ancajas (35) used acetylene reduction and ^{15}N method to study N_2 fixation in the rhizosphere of rice under field condition in the Philippines. They reported that nitrogen fixation ranging from 3 to 63 kg/ha occured in soil, water and the rice plant rhizosphere. The highest N_2 -fixing activity was found in the rhizosphere and was more pronounced under flooded than under upland condition. The nitrogen-fixing activity in the flooded soil increased after the panicle initiation stage of the rice plant. It was higher in the planted soil than in the unplanted soil. In 1978 , Hirota, et al. (27), collected 50 strains of rice from various countries in Asia. The plants were grown in pots until they flowered and the N_2-fixing activity of these intact plants were then measured by acetylene reduction method. A wide range of nitrogen fixing activity was observed. There were significant variations between strains. The highest value was about 1100 nmol per plant per hour. Strains showing high activity were collected in India and Thailand. They concluded that the rice plant genotype influenced the association with nitrogen-fixing bacteria in its rhizosphere.

In Thailand, Firth, et al., (36) studied the nitrogen balance in the rice paddies field of the central plane, they

suggested that biological N_2-fixation should account for the positive N_2-gained of 47 kg/ha in the non-fertilized plot. In 1974 Matsuguchi, et al. (15) investigated for the N_2-fixing potential in 40 paddy fields by collecting the top soil (0-2,cm) and paddy water samples consecutively through out a rice growing season. They used the dilution method to determine the abundance of N_2 fixer and the acetylene reduction method to measure the N_2-fixing activity associated with the soil and water samples. Blue-green algae, non sulfur purple bacteria, Clostridium butyricum, Beijerinckia sp. and Azotobacter sp. were the diazotrophs reported. The average amount of annual nitrogen fixed by these N_2 fixer as estimated from acetylene-reducing activities of soil and paddy water sample was 6.9 kgN/ha. During (1976-1978), Cholitkul, et al. (37) used in situ C_2H_2 -reduction technique to assess the effect of phosphorus on N_2-fixation activity associated with the rice paddies in Chainat, Supanburi, and Klong-laung. Assays were made at the maximum tillerimg, heading and maturing stages during four continuous cropping seasons spanning this 2 years. They reported the average of daily C_2H_2 -reduction activity at Chainat, Supanburi and Klonglaung were 0.83, 1.5 and 0.43 mmol C_2H_4/m^2 respectively in nonfertilized plot, and were 1.8, 2.1 and 0.92 mmol C_2H_4/m^2 respectively in PK-fertilized application plot. They concluded that P-fertilizer application enhance N_2-fixation without addition of nitrogen fertilizer.

In 1977, Simasatitkul and Boonjawat (38) studied biological nitrogen fixation in the rice paddy field by using an in vitro

acetylene reduction method. Rice (Oryza sativa varity RD.1) was grown in the nonfertilized plots at Klonglaung Rice Experiment Station, Pathumthani. Washed rice root, rhizospheric soil and paddy water samples were collected biweekly from these plots throughout the rice growing season. Comparison of nitrogen fixation rate in the 3 types of paddy showed that washed rice contributed the highest nitrogen fixing activity about 1000-lold of rhizospheric soil and paddy water. The N_2-fixing profiles are more or less similar either in the nonfertilized plots or NPK-fertilized plots, i.e. maximum activity resides in the panicle initiation stage. These results indicated that the dominant N_2 fixer are the rhizospheric bacteria.

The evaluation of the biological N_2-fixing activity in the rice paddies of Thailand in a uniform unit kgN/ha/crop is summaried in Table 1. The calculation is based on the following assumptions:

1. One mole of N_2 is reduced for every 3 moles of C_2H_2.
2. One hectare contains 1.6×10^5 rice hill.
3. 1 crop = 120 days.

Table 1. Evaluation of the N_2-fixing activity of the rice paddy fields in Thailand.

Authors.	Duration. (crop)	Method.	Site.	N_2 fixed (kgN/ha/crop)	
				Non-fertilized	Fertilized.
Firth et al. (36)	2	N-content	Chainat	47	
Matsuguchi et al (15)	1	C_2H_2 reduction in vitro (soil, water)	40 paddies	6.9	
Cholitkul et al. (37)	4	C_2H_2 reduction in situ	Chainat	9.3 †	20.2 *†
			Supanburi	16.8 †	23.5 *†
			Klonglaung	4.8 †	10.3 *†
Simasatitkul and Boonjawat. (38)	1	C_2H_2 reduction in vitro (washed rice root)	Klonglaung	6.6 †	6.0 **†

* PK = 38, 38 kg/ha

** NP = 74, 74 kg/ha

† calculated values.

5. This research.

The aim of this research is to promote the biologioal N_2-fixation in the North-Eastern part of Thailand. To approach this aim, we have conducted the following:

5.1 We have studied the N_2-fixation potential throughout one prowing season at three experimental sites, Tapra, Chumpae, and Rangsit. The first two sites are in Khonkan Province and the third in Pathumthani Province. In these three selected sites, we can study the rhizospheric N_2-fixing profiles under the influence of the following factors;

5.1.1 difference in soil type.

5.1.2 difference in fertilizer treatment.

5.1.3 difference in rice variety.

We have measured the N_2-fixing activity of the rice rhizosphere in varicus conditions at these three sites by using the in vitro acetylene reduction technique.

5.2 From the rice root and rhizospheric soil samples possesing ARA, we isolated the N_2-fixing bacteria using nitrogen free medium aerobic condition. We screened for heterotrophic diazotrophs having N_2-fixing activity higher than 1 umol/OD_{420}/d.

5.3 We have characterized these N_2-fixers by the following properties;

5.3.1 morphology characteristic

5.3.2 growth curve

5.3.3 effect of temperature on growth

5.3.4 effect of pH on growth.

5.3.5 relationship between N_2-fixing activity and stage of growth.

It is hoped that the information obtained from this research, although only basic in concept, but is necessary for the future implication of biological N_2-fixation in the rice paddies.