ROLE OF DIAZOTROPHS IN NITROGEN ECONOMY OF WETLAND RICE SOILS

A Thesis
submitted to the
Bidhan Chandra Krishi Viswavidyalaya
for the award of the Degree of Doctor of Philosophy
in
AGRICULTURAL CHEMISTRY AND SOIL SCIENCE

By

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2000

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This is to certify that the work recorded in the thesis entitled "**Role of diazotrophs in nitrogen economy of ~~low~~land rice soils**" submitted by Shri Ashok Choudhury for the award of the Degree of Doctor of Philosophy in Agriculture (Agricultural Chemistry and Soil Science) of the Bidhan Chandra Krishi Viswavidyalaya, is a faithful and *bona fide* research work carried out under my supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma. The assistance and help received during the course of investigation have been duly acknowledged.

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ACKNOWLEDGEMENT

I take this opportunity to express my deep sense of gratitude and indebtedness to Dr. M. C. Kabi, Reader (Research),Department of Agricultural Chemistry & Soil Science and Officer-In-Charge of the project of 'Survey, Selection & Mass Production of Nodule Bacteria', Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya for his keen interest, constant guidance and critical suggestions throughout the course of investigation and preparation of manuscript. Without his assistance and guidance, it would not have been possible for me to submit the thesis in its present form. I shall remain ever obliged to him.

Sincere appreciation are due to Associate Director of Research of Terai Zone, Hill Zone, Old Alluvial Zone, New Alluvial Zone, Red Laterite and Coastal Saline Zone of Bidhan Chandra Krishi Viswavidyalaya and successive heads of the Department of Agricultural Chemistry and Soil Science, BCKV, Mohanpur for their kind permission and providing necessary facilities for doing the experimental works.

I am also thankful to Dr. A. K. Saha, Convenor, Department of Agricultural Chemistry and Soil Science for providing me the laboratory facilities for this investigation.

I also acknowledge with thanks to Dr. K. C. Saha, Department of agricultural chemistry and Soil Science for providing me Gas Chromatography facility for my research work.

Sincere thanks are due to Dr. S. Kundu, Sri T. Biswas, Dr. S. Pal, Dr. P. K. Bandapadhaya, Dr. A. Bhattacharya, Dr. G. Pandit, Dr. B. Punja, Sri S. Basak and Other member of teaching and nonteaching staff of BCKV, B. N. Campus, Pundibari, Cooch Behar for their help, Suggestion and co-operation during the investigation.

Assistance rendered by Sri U. Ghosh in computerization of the thesis with meticulous care is also acknowledged.

Perhaps words are not enough to express my indebtedness to my parents, Mrs. N. Choudhury and other members of my family for their constant inspiration and encouragement, at times seemed to be highly indispensable to complete the research programme.

Mohanpur, Nadia
Date..1st February, 2000.

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LIST OF ABBREVIATIONS

ARA	Acetylene reduction assay
BNF	Biological nitrogen fixation
CCM	Combined carbon medium
CFU	Colony forming unit
cv.	Cultivar(s)
DAT	Days after transplantation
DW	Distilled water
FYM	Farm yard manure
hr.	Hour(s)
IAA	Indole acetic acid
JM	Jensen's N-free medium
MPN	Most probable number
Ndfa	Nitrogen derived from air
PCR	polymerase chain reaction
PGR	Plant growth regulators
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RRS	Regional research station

CHAPTER - I

INTRODUCTION

INTRODUCTION

Rice, the most important food grain crop, provides 20% of global human per capita energy and 15% of per capita protein. Rice occupied 148 million hectare, 10% of world's arable land, for a global production of 420 million tonnes (IRRI Rice Almanac, 1993). Against this global scenerio India produces about 100 million tonnes of paddy from over 42 million hectares of land. The productivity of rice in India is only about 1.9 tonnes /ha which is lower than the productivity of other Asian countries like Japan, Korea and China. With the exception of West Bengal, productivity of rice in eastern states, which accounts for 58% of the country's rice area, is still around 1.2 tonnes/ha (Siddiq,1991). The per capita availability of cultivated land in India is declined from 0.48 ha in 1951 to about 0.20 ha in 1981 and it is expected to decline further to only 0.14 ha by 2000 A.D. (Chaudhry and Sharma,1992). Thus when the population of India is galloping forward and size of land holdings are shrinking we have to produce the projected share of rice necessary for sustaining the present level of food sufficiency i.e. a steady increase in productivity from the present level of 1.9 tonnes/ha to 2.47 tonnes /ha and 3.5 tonnes/ha, respectively by 2000 and 2010 A.D. with least damage to the natural resource base (Siddiq, 1995). So, there is a need to generate high productive system to feed billion of peoples in the next century and like all other developing countries this would be the major scientific challenge for Indian agricultural scientist.

The high productivity of rice crop, achieved mainly through the introduction of fertilizer responsive high yielding varieties, should be sustained. In high productive system, nutrients, applied exclusively through inorganic sources, are known to increase yield initially at optimum doses of N P K, but the yields are not sustainable over the years. Continuous use of such

chemical fertilizers in tropical soil which, because of absence of organic matter, loose their natural fertility and required greater amount of fertilizer to produce the same yield. In addition to that high doses of N-fertilizers produced more nitrates than are absorbed by rice plant, the excess amount cause harm to aquatic animals and those who consumed the water charged with excessive nitrates. Another dimension of using higher doses of N-fertilizer is that our country is still deficient in nitrogen fertilizers. Projected data also indicates that by 2000 A.D. total requirement of N-fertilizers would be 18 million tonnes and available would be 13 million tonnes which is again deficient by 5 million tonnes. Thus use of higher doses of chemical fertilizer alone deteriorate both soil fertility and human health which eventually adversely affect economy and ecology of a region.

To over come these problems Integrated nutrient management (INM) would be of best help, which includes judicious use of organic, inorganic and biological resources (BNF) so as to sustain optimum yields, to improve or maintain the soil chemical, physical and biological properties and to provide crop nutrition packages which are technologically sound, economically attractive, practically feasible and environmentally safe.

Nitrogen is the most important nutrients in lowland rice based cropping system. Reduced fertilizer-N used in this cropping system, while maintaining or enhancing crop out put, is desirable from both environmental and economic perspectives. This may be possible by producing N on the land itself through biological nitrogen fixation. In wetland situation, flooding of rice soils changes the nutrient supply capacity, physico-chemical and microbiological properties of soil in such a way that all kinds of N_2-fixing organisms specially heterotrophic N_2-fixing bacteria can find conditions favourable for their growth. Since the establishment of the fact that heterotrophic BNF associated with rice has a substantial contribution to

lowland rice (Rinaudo *et al.*,1981 ; Watanable and Lin ,1984; Sethunathan *et al.*, 1983; Nayak *et al.*, 1986 ; Watanabe, 1986), the rice microbiologists are trying to exploit it for N-economy in wetland rice field.

The amount of heterotrophic nitrogen fixation in wetland rice soil vary with the methods used to estimate BNF. Estimates from N-balance in unfertilized planted pots averaged 7kg N/ha (App *et al.*,1980). With ARA, extrapolated values range from 0.8 to 6.0 kg fixed N/ha /crop cycle (Roger and Watanabe, 1986). Using N-dilution techniques it was estimated that BNF contributed 16 - 21 % of rice N or 11 - 16 Kg N/ha/crop (Hz *et al.* , 1986).

So , there remains ample scope of harvesting maximum benefit of N-fixation by heterotrophic bacteria in rice fields under low N-level. The idea would be of much interest particularly for small and marginal farmers who can hardly afford for higher dose of fertilization. Inspite of this information, various kinds of results, that include positive effect, inconsistent or negative effect were found when wetland rice were inoculated with selected N-fixing bacteria.

It has long been reported that most bacteria (80%) found in rice roots are N_2-fixing bacteria (Watanabe *et al.*, 1981). It is not that all diazotrophs found in rice root zone are effective N_2-fixers and colonize there effectively in root zone. Hence, effective diazotrophic strains should be isolated from the rice root and these will be screened for their effectivity and should be characterized prior to use as inoculant for rice.

Different rice germplasms, have different genetic background governing quality and quantity of root exudates and types of root system which ultimately regulate the colonization of roots by diazotrophs and the efficiency of N_2-fixers. This 'genotype x diazotrophs' interaction is again influenced by the environment specially the edaphic factors. Because of this

complex phenomena N_2-fixing system due to inoculation to a particular rice variety may or may not be operative efficiently. Hence, selection of rice 'variety x diazotroph' is an important aspect and it is to be considered to make an inoculation programme successful.

It is observed more frequently that in some locations bacterial inoculation has successful effect in improving the economic yield of crops, while in other location it fails. Though the N_2-fixing capacity of bacterial strains are their genetic character but their performance and efficiency solely depend on various edaphic factors. Hence, selection programme should be undertaken for selecting best adapted strain zone wise.

Unsuccessful biofertilizer treatment programmes are also very common due to improper inoculation method ; for instance seed inoculation technique as adopted in case of legume crops does not work effectively in case of rice seeds. Another dimension of inoculation technique is the use of mixed inocula. It is to be tested that the hypothesis of superiority of mix inocula due to the positive metabolic association among the strains may help in the establishment of diazotrophs, and it also bring down the possibility for failure of more strains at a time.

Nitrogen fixation is an energy requiring process and need much energy for breaking the triple bond of molecular nitrogen. Hence, for heterotrophic nitrogen fixer available carbon source is much essential for maintaining their growth as well as efficiency. But in tropical climate, soil being less in organic matter sometimes does not always support the growth of heterotrophic N_2-fixer in rice root zone and ultimately lead to the failure of biofertilizer programmes. To overcome this constrain sufficient quantity of easily decomposible organic matter should be added to make the inoculum. affective.

Integrated nutrient management system in rice culture include the application of both inorganic nitrogen and biofertilizer. Various reports indicate that biofertilizer may not be much effective at very low as well as very high N-fertilizer application and this also varies with the nature of soil. Hence, it would be a wise scientific step to select the correct dose of N which does not suppress the N_2-fixation and at the same time provide economic yield return at various agroecological zones. Keeping the above facts in view, the present investigation has been taken up with the following objectives :

(i) To isolate diazotrophs from rice root zone and screening these bacteria for their N_2-fixing capacity and ability to produce IAA.

(ii) To characterize the isolated diazotrophic bacterial strain.

(iii) To screen the effective 'diazotroph x cultivar' combination.

(iv) To study the performance of diazotrophs at different agroecological zones of west Bengal .

(v) To study the effect of different inoculation method on the effectivity of biofertilizers.

(vi) To assess the performance of mixed inocula over single one.

(vii) To study of role of organic mater on the effectivity of biofertilizer.

(viii) To assess the performance of biofertilizer under graded levels of N-fertilizer at various agroecological zones of west Bengal.

CHAPTER - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Nitrogen fixation by free living bacteria associated with plant has been universally accepted today, although it remains to be fully exploited. Rice, one of the most important crop plants is known to support such biological nitrogen fixing activity. The presence of N_2- fixing bacteria in rice rhizosphere was reported as early as 1929 (Sen, 1929) perhaps this is the first report of nitrogen fixation in the rice rhizosphere, but this observation was over looked. Then it was further recognized by De in 1939 that the natural fertility of tropical rice field was due to the biological nitrogen fixation by Cyanobacteria. In 1962, Dobereiner and Ruschel studied the growth stimulation of N_2-fixing bacteria in the rhizosphere of lowland rice. Their observations led to Dobereiner's idea that non- nodulated, non-leguminous plant can fix atmospheric N_2 through bacteria associated with roots (Dobereiner and Day, 1975). Yoshida and Ancajas in 1971 first demonstrated N_2- fixing activity of wetland rice roots by using sensitive acetylene reduction assay.

2.1. Rice rhizosphere and diazotrophic rhizobacteria

Rice plants grown in flooded soil have a unique biochemical characteristic. The ability of rice roots to function efficiently in anaerobic condition is one of the most interesting and important aspects of rice culture. Some workers (Armstrong, 1960) has shown that the rice plant supplies molecular oxygen to the root and the air transporting system of the rice plant develops to a greater extent under flooded condition than under upland conditions (Arashi, 1959). Because the total surface area of plant roots is much larger than the soil surface occupied by the plants the root-soil

boundary in flooded soil may be an important area for aerobic metabolism of nitrogen fixing aerobic bacteria. Moreover, such flooding changes the chemistry, microbiological properties and nutrient supplying capacity of the soil. As a result, all kinds of N_2-fixing organisms including aerobic, microaerophilic, facultative anaerobic and anaerobic bacteria can find conditions favourable for their growth in rice soil and in association with rice roots.

In order to support energy expensive biological nitrogen fixation in the root region, rice plant release large amount of organic compounds including different amino acids and carbohydrates (Rovira, 1965). This wide range of carbohydrates serves as carbon and energy sources for a variety of diazotrophs in rice rhizosphere. A list of diazotrophs found in rice rhizosphere and rice root is given below :

Diazotrophs in root and rhizosphere of rice :

Name of Diazotrophs	References
Aerobic Organisms	
Azotobacter spp.	Bhattacharya (1958), Rangaswami and Subbaraja, (1962)
Beijerinckia spp.	Dobereiner and Ruschel, 1962
Microaerophilic Organisms	
Azospirillum spp.	Lakshmi Kumari *et al.* (1976), Nayak and Rao, (1977), Watanabe *et al.* (1979), Ladha *et al.* (1982).

Name of Diazotrophs	References
Pseudomonas spp.	Watanabe and Barraquio, (1979), Watanabe *et al.* (1982), Barraquio *et al.* (1983).
Pseudomonas diazotrophicus sp. nov.	Watanabe *et al.* (1987).
Alkaligenes faecalis	You *et al.* (1983). Qiu *et al.* (1981).
Facultative Organisms	
Klebsiella pneumoniae	Watanabe and Barraquio (1979)
Klebsiella planticola	Ladha *et al.* (1983)
Klebsiella oxytoca	Yoo *et al.* (1985).
Enterobacter spp.	Watanabe *et al.* (1979) Ladha *et al.* (1983)
Enterobacter cloacae	Qiu *et al.*, (1981) Ladha *et al.* (1983)
Anaerbic organism	
Clostridium spp.	Hirota *et al.* (1978).

Because of the generic diversity, many different isolation media and conditions are required to count and isolate N_2-fixing bacteria from rice roots. To overcome this problem of the N_2-fixing microflora of a rice rhizosphere was studied by a new enrichment and isolation procedure, where the exudates

from a germinating seed ("the spermosphere model") were used in "C " & "N" free medium (Bauzon *et al.*, 1982). In this study, " the spermosphere model" gave N_2-fixing isolates with a frequency of 65% of the total microflora. Thirty two of the many isolates obtained have been studied in detail - these were *Klebsiella oxytoca, Enterobacter cloacae, Pseudomonas paucimobilis* and *Azospirillum.* All these bacteria were present at densities higher than $10^5 g^{-1}$ of dry soil in the initial rice rhizosphere. Another attempt was made by Ueda *et al.* (1995) to overcome any bias from culture dependent method of studying rhizospheric diazotrophs. In this method *nif* D segments from crude rice root DNA were amplified by the polymerize chain reaction (PCR) and *nif* D library was constructed. Sixteen cloned *nif* D genes chosen at random from the library were sequenced. A comparison with published sequences indicated the presence of seven noble groups of *nif* D proteins, which implies the existence of at least seven components in the diazotrophic community of rice roots, dominated mainly by proteobacteria. However, no *Azospirillum* like *nif* D was found, although many reports indicated the widespread presence of *Azospirillum* species. Therefore, it is still not clear whether *Azospirillum* species are the widespread N_2 - fixing bacteria in rice roots.

2.2 Population dynamics of diazotrophic bacteria associated with rice

After the discovery of BNF in rice rhizosphere it had been the key interest of the workers to investigate the population dynamics of various N_2-fixers in rhizosphere, rhizoplane, histosphere and stem of rice at different growth stages under dryland and wetland situation. As early as 1978, Watanabe and Barraquio observed that inner rhizoplane (histosphere) has

more heterotrophic bacterial population than rhizosphere, rhizoplane and stem of rice. They also reported the percentage of bacterial isolates providing nitrogenous positive was 81% for inner rhizoplane bacteria,76% for rhizoplane bacteria, 37.5% for bacteria from stem and 2.4% for rhizosphere soil bacteria. In the same laboratory (Watanabe *et al.*, 1979) the population of aerobic heterotrophic N_2-fixing bacteria associated with rice roots and stem was determined by the MPN method, using semisolid glucose yeast - extract and semisolid malate yeast extract media. The glucose yeast- extract gave 10 to 100 times higher count of aerobic N_2-fixing bacteria associated with rice roots than did the malate yeast - extract media, on which *Azospirillum* like bacteria were usually observed. Aerobic heterotrophic N_2- fixing bacteria of 3 varieties were also compared at heading stage. Although the rice cv. IR-34 showed the highest population of N_2-fixing bacteria on the rhizoplane in glucose - yeast - extract medium, it had the lowest population in the histosphere. Another cultivate, IR-32, which had the lowest field ARA activity, did not give the lowest MPN in either the rhizoplane or the histosphere.

Growing situations also affect the dinitrogen fixing bacteria associated with rice at various growth stages (Barraquio and Watanabe, 1981). All the root tissues of wetland plants yielded high percentage of N_2-fixing bacteria ranging from 20 to 90% of the total aerobic heterotrophs. Other portions of the plants such, as the stem and leaf sheath were inhabited by N_2-fixing bacteria to a lesser extent. In the dryland rice the N_2-fixing bacteria were 15 - 30% of total aerobic heterotrophs. No *Pseudomonas* were isolated from dryland rice, where as , it was predominant in wetland rice ranging from 26 - 93% of the total aerobic heterotrophic N_2-fixers . High number of *Pseudomonas* in rice rhizosphere was also reported by Vlassak *et al.*, (1992).

Barraquio *et al.*, (1982) also reported similar findings with rice varieties IR 5 and OS 4. Population of N_2-fixing microorganism on glucose yeast - extract and malate yeast - extract media were 10 times higher in wetland than that of dryland rice. The higher number of nitrogen fixing bacteria in wetland condition was found in all three sites - rhizosphere soil, histosphere and basal shoot. (Barraquio and Watanabe, 1981; Barraquio *et al.*, 1982) pointed out that the lower N_2-fixing activity of dryland rice was related to the lower number of N_2-fixing bacteria associated with the root and basal shoots. If the lower nitrogen fixing activity of dryland rice were due to the difference of the root in exudate, there should be a difference in the number of heterotrophs, but this was not the case. Thus it seems logical to believe that there must be favourable conditions in wetland root for the selective development of N_2-fixing bacteria.

Recently, Van Holm *et al.*, (1994) observed that the number of dinitrogen fixing bacteria in the rhizosphere of the three rice cultivars was not significantly different. They also reported that the population of diazotrophs at all plant development stages measured and the number of *Pseudomonas* was found to be significantly higher than *Azospirillum* and *Klebsiella*. The number of N_2-fixing bacteria increased until tillering stage and decreased thereafter although highest nitrogenase activity was observed at heading stage (Roger and Ladha, 1992). Ladha *et al.*, (1986), using MPN technique, measured the population of N_2-fixing aerobic heterotrophs after incorporation of straw. The results with a higher number of N_2-fixing bacteria on decomposing straw pieces and a significant positive correlation between ARA and dry weight of straw obtained from straw treated soil, confirm the finding of Wada *et al.*, (1979). They reported that organic debris were good microsites for N_2-fixation.

The ability of heterotrophic N_2-fixing bacteria to grow chemolithotrophically on $H_2 + CO_2 + O_2$ or $O_2 + CH_4$ (Hanus *et al.*, 1979; Pedrosa *et al.*, 1980 ; Sampaio *et al.*, 1981) was known. Since wetland rice soils (Yamane and Sato, 1964) and wetland rice root (Kimura, 1981) are known to evolve H_2 gas, it had been a point of interest that whether N_2-fixing bacteria in the roots of wetland plants (or on the surface of root) have an ability to use H_2 and grow autotrophically on H_2. Probably, the first report on this aspect published in 1982 by Watanabe *et al.*, The aerobic N_2-fixing bacteria were isolated from wetland soil, rhizosphere soil, root and basal shoot of wetland rice, dry land soil and root of dry land rice. N_2-fixing bacteria capable of autotrophic growth under on $H_2 + CO_2 + O_2$ were found almost exclusively from the rhizosphere and the root of wetland rice. They also found update of hydrogenase activity by all the on N_2-fixing bacteria isolated from wetland rice root.

The incidence of H_2-oxidizing chemolithotrophic bacteria associated with rice grown under continuous wetland, upland and rainfed wetland condition was studied by ^{14}C - autoradiographic technique in a neutral soil at IRRI (Maahas) and an acid rainfed soil (Luisiana) (Gowda and Watanabe, 1985). In Maahas soil, H_2 -oxidizing chemolithotrophic bacteria were not detected in the endorhizosphere, rhizosphere and nonrhizosphere soil of rice under dryland conditions. Under continuously flooded conditions a very large population of these bacteria were found in the endorhizosphere but not in the oxidized and reduced soil. Gowda and Watanabe (1985) further studied the H_2 -supported N_2 -fixation of *Pseudomonas sp.* and *Azospirillum lipoferum.* Hydrogen - dependent chemolithotrophic nitrogen fixation by *Pseudomonas* sp. strain H8 and KLH76 and *Azospirillum lipoferum* strain 341 was confirmed by acetylene - reduction assay (ARA) and ^{15}N incorporation.

Glucose at lower than 2mM did not significantly repress H_2-dependent acetylene reduction of chemolithotrophically grown *Pseudomonas* sp H_8 and *Azospirillum lipoferum*. From these findings it appears that H_2 - dependent ARA is active in carbon limited condition and at low O_2 level.

2.3 *In vitro* selection of diazotrophic strains and rice cultivars

Selection or improvement of N_2-fixing bacterial strains is one of the possible ways to increase the efficiency of biological nitrogen fixation system. In selecting the most efficient combination of a N_2-fixing bacterial strains isolation was made from the rhizosphere of actively N_2-fixing rice plants. Isolated strains were then tested with rice cultivars using the spermosphere model in which an axenic rice seedling was grown in darkness in a Pankurst tube on a medium without 'C' and 'N' (Thomas - Bauzon *et al.*, 1982). This system provided a means to test bacterial strains with rice exudates as the sole C source. The atmosphere of the tubes contained 1% acetylene and each "strain & cultivar" combination was characterized by its maximum ethylene production using 15-20 replicates. This approach was used by Charyulu *et al.*, (1985) to screen seven strains of bacteria and seven rice varieties. The association, considered most efficient (*Azospirillum lipoferum* 4B x rice cesario M_7) was tested in the field, but differences in yield appeared to be erratic in their independent field experiments (+27 % with no N, -28 % with 92 kg/ha of N, + 43 % with 120 kg/ha of N and - 4 % with 150 kg/ha of N). Omar *et. al.*, (1989) used a similar approach and reported significant yield increases, ranging from 6 to 21% in field experiments in Egypt. Higher increases in yield were obtained at higher N levels (76 - 96 kg/ ha of N). The approach to improve the effects of inoculation used by Charyulu *et al.*, (1985) and Omar *et al.*, (1989) considered both the soil to be inoculated and the rice variety to be grown for selecting the most efficient strain. The potential for practical

utilization of this method strongly depended on the degree of specificity required to select an efficient bacterium for a given agroecological condition (Roger, *et al.*, 1993).

2.4 Methods of inocultion and concept of mixed inoculum.

Different inoculation methods are adopted to make the inoculation effective. Inoculation of rice are generally performed by dipping seeds or coating them with various carriers, dipping seedlings in bacterial cultures, nursery soil inoculation , field inoculation and foliar application. Little attention has been paid, however, to the relative efficiency of these methods. In a comparison of seven combinations of seeds, seedling and soil inoculation, a significant increase in yield was obtained only when seedlings and soil were inoculated (Gopalaswamy and Vidhyasekaran, 1987a, b). Foliar application of *Azotobacter* increased yield by 7.5% without N fertilizer and by 3.7% with 50 kg / ha of N (Kannaiyan et al. 1980)

One important aspect of inoculation to make it effective, is the use of mixed a inoculum. A better response to inoculation was observed when *Azotobacter* was combined with P-solubilizing bacteria (Kundu and Gaur, 1984). Dewan and Subba Rao (1979), however, observed that dual inoculation with *Azospirillum brasilense* and *Azotobacter chroococcum* gave lower (26%) increase in rice yield than single inoculations (49 and 62%, respectively).

Hamza *et al.*, (1993) reported on the possible interaction among various genera of diazotrophs when grown in mixed batch cultures in presence of carbon sources available in pure form or in root extract. Results indicated a synergistic effect among various species as doubling time decreased by 4.7, 2.3 and 1.4 folds for *Pseudomonas putida, Klebsiella pneumoniae* and *Azospirillum brasilense*, respectively. Results of pot

experiments demonstrated that inoculation with tested diazotrophs particularly their mixed population ,did improve the biomass production of barley and wheat. Abbas *et al.*, (1993) also observed increased grain yield and total day matter yield of various wheat cultivars with residual dose of nitrogen and mixed inoculum of *Azotobacter* sp., *Azospirillum* sp. , *Klebsiella* sp., *Pseudomonas* sp. and *Bacillus* sp.

The basis of the synergistic or inhibitory effect of mixed culture is not still clear. Nitrogenase activity (ARA) of pure cultures and coculture of different *Azospirillum* species (A. *brasilense,* A. *lipoferum,* A. *irakense*) and of *Bacillus polymyxa* was measured in association with rice using the spermosphere model (Khammas *et al.*, 1993). Ethylene was produced more rapidly by the cocultures than by the pure cultures. Mean values of ethylene production by the cocultures (1930 n moles C_2H_4 / plant / day) were significantly higher than that obtained with pure culture (790 n moles C_2H_4 / plant / day). Coculture between *A. brasilense* or *A. lipoferum* and *B. polymyxa* gave high nitrogenase activity values, whereas, association between *A. irakense* and *B. polymyxa* did not lead to increased nitrogenase activity. They suggested that in the spermosphere model, two polysaccharides could serve as carbon sources for the *Bacillus* strain, starch of the seed and the root mucigel. The cocultures can be considered as metabolic associations where the *Bacillus* strain provides degradation and fermentation products which can be used by *Azospirillum.* Cocultures enable the utilization of polysaccharides for nitrogen fixation, which are usually bad substrates for single culture.

2.5 Establishment and persistence of inoculated strains

Little information is available on the establishment of inoculated strains. Studies on *Azotobacter* reported better establishment in the rhizosphere of plants grown in sterile than in nonsterile soil, with no effect on

soil microbial populations (Gopalakrishnamurthy *et al.*, 1967; Neelkantan and Rangaswami, 1965; Purushothaman *et al.*, 1976). No statistical analysis of the data was made , but the range of variations in the number of microorganisms per gram of rhizosphere soil observed were most often lower than one to five and, considering the low accuracy of dilution techniques, differences were probably not significant in many cases. Watanabe and Lin (1984) found higher count of N_2-fixing bacteria at maximum tillering, early flowering, and late flowering in the roots of rice inoculated with *Azospirillum* or *Pseudomonas*, but the difference was considered significant only at early flowering stage, particularly in the root washings (rhizoplane) when N_2-fixing populations were more than ten times higher in inoculated plants than in the control. They also observed that after dipping the root for one day in a bacterial suspension of 10^8 cells / ml, 10^3 to 10^4 cells of bacteria per rice seedling were adsorbed by the root. Balasubramaniam and Kumar (1991) reported the five times increase of *Azospirillum* population in inoculated rhizosphere sample over control at 50%, 75% and 100% N level.

With the use of a marker strain of *Azospirillum lipoferum*, resistant to streptomycin and rifampicin, Nayak *et al.*, (1986) found survival but no establishment of the strain. In one experiment, the strain disappeared within one month, whereas, in a second experiment, it survived in the soil, the rhizosphere, and on the stems for 50 - 70 days without any increase in number. The greatest density of inoculated *Azospirillum* (5.3×10^4 / g dry weight of root) was recorded in the rhizosphere 40 days after transplanting whereas, putative indigenous populations of *Azospirillum* were about 500 times larger (2.8×10^6 / g dry weight of root). Despite the low level of inoculated *Azospirillum*, inoculation increased the dry weight and total N of the plant. The only information on long-term effect of inoculation is a report

by Balasundaram and Sen (1971) showing no residual effect of rice inoculation on a succeeding wheat crop.

Few of the mechanisms involved in the colonization of rice root by inoculated bacteria have been studied so far. Balandreau and Knowles (1978) and Diem *et al.*, (1978) reported a significant role of mucigel in rice root colonization by *Beijerinckia* . Murty and Ladha (1987) observed that root colonization by *A. lipoferum* was unaffected by root mucigel and instead suggested that colonization was related to the nature of the root surface and the presence or absence of mucigel. Tabary *et al.*, (1984) isolated and purified a lectin from rice embryos that interacted in vitro with different bacteria isolated from the rice rhizosphere. The most efficient binding was observed with a strain of *Beijerinckia.*

Little is known about the competition between indigenous and inoculated strains. Rai (1985) observed that mutants of *Azospirillum*, resistant to the herbicide, machete, increased yield more efficiently in a machete - treated soil (+ 44%) than the parent strain in a nontreated soil (+5%).

2.6 Mode of action of inoculated bacteria on rice

The beneficial effect of bacterial inoculation can be attributed to a combination of increased associative biological nitrogen fixation in rhizosphere, production of PGRs that favour rice growth and nutrient utilization, increased nutrient availability through solubilization of immobilized nutrients by inoculated bacteria, and competition of inoculated strains with pathogens or detrimental bacteria in the rhizosphere. However, the relative importance of these four components has not yet been determined.

Current estimates of biological N_2 fixation in rice rhizosphere are not sufficient to explain the increase in yield reported in the literature. If one assumes that all N_2 fixed is absorbed by the plant, the average increase in yield of about 0.5 tons/ha reported in field experiments would require at least an increase of biological N_2 fixation by 10 kg/ha per crop. (Roger *et al.*, 1993). But no available data demonstrate a marked and durable increase of biological N_2 fixation in inoculated rice. Watanabe and Lin (1984) recorded a significant increase in ARA at flowering stage in rice inoculated with *Pseudomonas* or *Azospirillum*, but it was equivalent to an increase of about 6.5 mgN per plant over 100 days. Thus, ^{15}N content of inoculated and control plants did not differ . Nayak and associates (1986) found that inoculating with *A. lipoferum* did not increase associative biological N_2 fixation (estimated by ARA, ^{15}N and N- balance studies) but did increase tiller number and early reproductive growth. These results indicate that the beneficial effect of the inoculum may not be related solely to N_2 fixation.

In inoculation experiments the benefit due to the combined application of fertilizer N and *Azotobacter chroococcum* and *Azospirillum* sp. was normally compared with different levels of fertilizer N alone (Shende and Kokorina, 1964 ; Kannaiyan *et al.*, 1983 ; Bagal and Patil, 1984) or in combination with the inoculum would alter the growth pattern and physiology of the rice plant as well as the plant associated microbial activities. Watanabe and Lin (1984) and Nayak *et al.*, (1986) observed enhanced uptake of mineral N by the rice plant occurred following inoculation with *A. lipoferenm.* Omar *et al.*, (1989) also considered that inoculation of *Azospirillum* should not be regarded as a substitute for N fertilizers and a technology for low - input rice cultivation, but rather, as a means to extend the uptake of biologically fixed N even at high level of N- fertilizers leading to maximization of crop yield. Recent evidence suggests that plant growth

promoting factors produced by the introduced microorganisms might improve yields to a considerable extent (Venkataraman and Neelakantan, 1967; Tien *et al.*, 1979; Venkateswarlu and Rao, 1983; Nayak *et al.*, 1986), but the evidence is not universal.

Few recent works provide information about the proton efflux and redox properties of rice roots due to inoculation with diazotrophic bacteria. Lin *et al.*, (1992) reported that inoculation of rice seedlings with *Alcaligenes faecalis* increased proton efflux from the roots within 6 hrs. Coating seeds with bacteria increased proton efflux more than soaking seedlings roots in bacterial suspension. *A. faecalis* increased ATP content of the rhizosphere, increased the solubility of $FePO_4$ and the availability of Fe in the rhizosphere and stimulated plant growth. Lin and You (1994) also reported that the same nitrogen fixing bacteria *A. faecalis* strain A1501 enhanced the redox ability of rice roots, stimulated superoxide dismutage activity and increased seedling resistance to high and low temperature. Inoculation with this bacteria increased polyphenol content in rice seedlings, and extracts of polyphenolics from inoculated rice roots stimulated N_2 - fixation and chemotaxis in *A. faecalis.*

2.7 General effect of inoculation

The analysis of data from 23 articles reporting 210 trials showed average increase in yield of 19.8%, but the response varied from - 33% to 1.25%. Average increase was higher in pots (27.6%) than in the field (14.0%) (Roger *et al.*, 1993). Average yield increase in field experiments (14.0%) was close to the minimum detectable difference (14.5%)which can be expected from the most commonly used experimental design ($16m^2$ plot with 4 replicates) (Gomez, 1972). His results indicated that inoculation did not invariably increase the yield. Different investigators found (1) no effect

(Rajagopalon and Rangaswami, 1988), (2) inconsistent effects (Maskey, 1976; Rao *et al.*, 1983) and (3) negative effects of inoculation. Data of a few pot and field experiments are available on the effects of inoculation with *Azotobacter* sp. *Azospirillum lipoferum*, *A. brasilense*, *Pseudomonas* etc.

The benefit of inoculation with *Azotobacter chroococcum* has given positive responses in green house and field experiments (Shende and Kokorina, 1964; Shende, 1965), however, other studies on inoculation with *Azotobacter* failed to show a yield response (Nair *et al.*, 1972; Bagal and Patil, 1984; Kavimandan, 1986). *Azotobacter* inoculation alone gave higher yield than application of 50 kg N / ha (Patil *et al.*, 1976; Mohammad, 1987). Reddy *et al.*, (1987) reported that use of *Azotobacter* and 50 kg N / ha resulted in 15 to 18 per cent increase in grain yield. Rangaswami (1975) reported that the nitrogen requirement of paddy could be reduced by 20 - 40 kg N / ha by *Azotobacter* inoculation.

Inoculation with *A. lipoferum* and *A. brasilense* isolated from rice roots and soils increased grain yield of rice. Watanabe and Lin (1984) reported that inoculation with *A. lipoferum* and *Pseudomonas* sp. promoted early tillering and reproductive growth of wetland rice. It also significantly increased the filling rate of grain and grain weight per plant at harvest. Rinaudo *et al.*, (1981) tested *Azospirillum* sp. isolated from rice and found differences in plant response to different isolates. Rao *et al.*, (1983) observed a statistically significant positive yield response to inoculation with *Azospirillum* sp. at different levels of N fertility over three consecutive seasons. The response to *Azospirillum* was more pronounced at 30 and 45 kg / ha of fertilizer N than at a higher level. Although the mean yield of both grain and straw were increased by inoculation with *Azospirllum*, the interaction between N fertilizer and inoculation was not statistically significant. Jeyaraman and Purushothaman (1988) reported positive effects of *Azospirillum* inoculation in

the absence of fertilizer and when N was applied at a rate of 50 kg / ha , but also a statistically significant decrease in yield by 25% was obtained in plots where N was applied at a rate of 75 kg / ha. Similarly, in a field experiment with two rice varieties, *Azospirillum* inoculation significantly increased yield by 19 and 43% without N fertilizer, but significantly decreased yield by 33% and 31% with 92 kg/ha of N (Charyulu *et al.*, 1985). This was attributed to enhanced denitrification by the added bacteria. Gopalaswami *et al.*, (1989) reported that *A. lipoferum* inoculation to soil fertilized with 75 kg N/ha significantly increased the grain yield. But at lower (25,50 kg N/ha) or higher (100 kg N/ha) N level, the bacterial inoculation did not have any significant beneficial effect. Heulin *et al.*, (1991) observed that under high productivity (equivalent to 10 t/ha for the control treatment at 76 kg N level), the inoculation of *A . lipoferum* had a positive, significant and increasing effect on grain yield +6% without N, +16% with 33 kg N and +12% with 76 kg N.

Associative biological N_2 fixation seems to be less sensitive to N fertilizer application than other N_2- fixation systems (Roger and Watanabe, 1986). This might explain why almost any kind of trend in the response of rice to inoculation at various levels of N fertilizer has been reported. Some reports indicate a better effect of inoculation with *Azotobacter* and *Azospirillum* without N fertilizer (Dewan and Subba Rao, 1977) or at low N levels (Balasubramanian and Kumar, 1987). Jalapathi Rao *et al.*, (1977) observed a negative correlation between N level and effect of inoculation with *Azotobacter*. Several reports indicate that the effect of inoculation was lower at no N fertilizer or at high levels of N fertilizer than at low or medium levels (Balasubramanian and Kumar, 1987 ; Balasundaram and Sen, 1971 ; Rao *et al.*, 1983). There are also reports of significant effects of inoculation with Azospirillum at high N levels (Charyulu *et al.*, 1985; Omar *et al.*, 1989 ; Heulin *et al.*, 1991).

2.8 Nitrogen fixation associated with organic matter breakdown.

Crop residues are basically celluloses, hemicelluloses and lignins, and these are the major energy source for metabolism of microrganisms. Biological nitrogen fixation is a energy rich biological process of N_2-fixing organisms and for heterotrophic N_2-fixation available 'C'-source is the major limiting factor in most of the soils. Substantial gains in N_2-fixation following the addition of 'C'- substrate were observed in flooded paddy soil (MacRae and Castro, 1967; Rice *et al.*, 1967; Rice & Paul , 1972 ; O'Tool & Knowles, 1973 ; Rao , 1978). It has also been established that anaerobic decomposition products of the organic matter leads to the substartes which would be directly available to the N_2-fixing organisms (Mac Rae and Castro, 1967; Rice and Paul, 1972). Moreover, anaerobic decomposition of rice straw and cellulose results in the accumulation of organic acids and alcohols that have been shown to stimulate N_2-fixation under submerged conditions (Rao, 1978). Addition of straw also caused a decrease in the inorganic nitrogen and in redox potential , making a favourable environment for nitrogen fixing bacteria. But the stimulation of nitrogen fixation was observed only when the soils were kept water logged (Yoneyama *et al.*, 1977).

Rao (1976 and 1978) has reported that 1.7 to 7.0 mg N is fixed per g straw incorporated into soil. Charyulu and Rao's (1981) data indicated this value can range from 0 in acid saline soil to 1.6 mg in acid sulphate soil . Durbin and Watanabe (1980) obtained ^{15}N incorporation corresponding to 1.3 mg N / g straw. All these reports were based on laboratory studies with high amount of straw (more than 1% of the dry soil weight). Ventura *et al.*, (1986) added straw @ 0.3% of the dry soil weight - that may be closer to the amount incorporated under field condition and observed 2 - 4 mg N is fixed per g of straw. Charyulu *et al.*, (1981) observed that rhizosphere samples from rice straw amended (@ 3 and 6 tons / ha) soil exhibited more pronounced

nitrogen fixing activity than the samples from unamended soil, while the activity of the rhizosphere samples from soil receiving combined nitrogen (40 and 80 kg / ha) was relatively low. however, the inhibitory effect of combined nitrogen was not expressed in the presence of rice straw at 6 tons / ha.

The general ability of diazotrophs to utilize straw and its breakdown products was also examined by inoculating representative strains of 9 diazotrophic genera onto straw, xylan, cellobiose and glucose in sand cultures. (Gibson *et al.*, 1988). All strains were able to utilize one or more of these substrates to support nitrogenase activity, although in some cases there was variability within a genus. *Azospirillum*, *Azotobacter*, *Azomonas*, *Bradyrhizobium* and *pseudomonas* were able to use straw in pure culture whereas, *Bacillus*, *Beijerinckia* and *Herbazospirillum* only did so when inoculated with *Cellulomonas* sp CSI-17. *Klebsiella*, although using straw to support respiration, failed to fix N_2 with or without *Cellulomonas* present.

The concentration of organic matter and its quality also determine the extent of N_2-fixation . Rao (1976) observed that under flooded condition, the addition of 1% straw to soil caused a significant increase of N_2-fixation, whereas with 2% straw the N_2-fixation was relatively retarded. In moist non-submerged conditions, however, more N_2-fixation occurred in soil amended with 2% straw than in soil amended with 1% straw. The lower values of N_2-fixation at 2% than at 1% of rice straw noticed only under flooded condition could be attributed to the formation of toxic products during the decomposition of higher quantities of straw under predominantly anaerobic condition of flooded soil system (Patrick, 1971). O'Toole and Knowles (1973) also reported that high concentration of glucose or mannitol (3%) inhibited C_2H_2 reduction in soil and attributed this to a drop in pH. Charyulu and Rao (1981) observed greater N_2-fixation occurred in submerged

soils amended with cellulose and rice straw, the former being superior. Addition of sucrose, glucose and malate in that order stimulated N_2-fixation in submerged alluvial soil, while sucrose alone enhanced N_2-fixation in laterite soil. In submerged acid soils none of these 'C'-sources stimulated N_2-fixation.

2.9 Rice varietal difference in promoting associative biological nitrogen fixation

Discovery of N_2-fixation in the rice rhizosphere open different frontier areas of biological nitrogen fixation research with rice plant. One of the major interest of soil microbiologist world wide was to find out '*nif* supporting traits among the rice cultivars with the objective to undertake breeding programme for rice plant that stimulate better N_2-fixation.

There are several reports on the existence of varietal differences in the ability to support associative biological N_2-fixation. Though nothing is known about the physiological basis of the apparent varietal differences in nitrogen fixation, a readily available source of energy excreted by the root system of rice specially carbohydrate and amino acid (MacRae, 1967) might play crucial role in establishing the association of diazotrophic microorganisms with rice. Nitrogen fixing microbes generally take advantage of this to grow and fix atmospheric nitrogen in condition which do not repress nitrogenase synthesis.

Probably the first report on this aspect came in 1978 by Hirota *et al.* They conducted experiment with fifty rice varieties collected from various parts of tropical Asia and nitrogen fixing activity of these varieties were measured. They suggested that the rice plant genotype influences the association with N_2-fixing bacteria in its rhizosphere. The variation among stains was statistically significant. The activity expressed per gram of dry root per hour also showed a wide range of significant variation between the

strains. There was a correlation (r = 0.895, significant at the 1% level) between root weight and nitrogen fixing activity within a strain. This means the larger the amount of root per unit area, the higher the activity. The highest value was about 1,100 n moles/plant/hr. In this experiment, strains showing high activity were collected in India and Thailand. Similar type of work was reported by Habte and Alexander (1980). They observed that N_2-fixation in the rhizosphere varied significantly among the 16 rice varieties. In tests of two varieties with dissimilar rates of nitrogen fixation in their rhizosphere, the variety which had the greater root weight and lesser shoot weight and which supported better methane formation had the greater nitrogenase activity.

Gilmour *et al.*, (1978) compared cultivars of different introduction dates, the nitrogenase activities of older cultivars introduced before the advent of inorganic N- fertilization were higher than or equal to the nitrogenase activities of those cultivars introduced later. The nitrogenase activities in the rhizosphere of wild, trisomic and cultivated rice species and in root associated *Azospirillum* sp. were evaluated under uniform field conditions (Patnaik *et al.*, 1994). A wide variation in rhizosphere nitrogenase activity was observed among the wild species of rice. This activity was low initially and reached a maximum during the heading and the reproductive phases of the rice plant growth cycle. *Oryza latifolia* exhibited low nitrogenase compared to the 6 other wild species and the trisomic rice plants exhibited greater nitrogenase activities relative to normal cultivated rice plants. Similarly, *Azospirillum* sp. isolated from the trisomics, exhibited greater nitrogenase activity relative to isolates from normal rice plants. Although *O. latifolia* had low rhizosphere nitrogenase, the *Azospirillum* sp. that was isolated from its rhizosphere had the greatest nitrogenase activity. In general, cultivated and trisomic rices

supported considerable nitrogenase activity in the rhizosphere and harboured most efficient *Azospirillum* sp. relative to wild rice species.

Although Hirota *et al.*, (1978) observed significant correlation between the root dry weight and nitrogen fixing activity within a rice strain, Sano *et al.*, (1981) from the same laboratory reported the absence of correlation between acetylene reduction activity and root dry weight, but among 47 *Oryza sativa* strains, acetylene reduction per plant was significantly correlated with acetylene reduction per g dry root ($r = 0.68$, $P<0.01$). In cultivated rice species, the mean specific activity of 47 *O. sativa* strains was 861 n moles/h/g dry root and that of 10 *O. glaberrima* was 780 n moles/h/g dry root though a wide range of variations was found within each species. On the other hand wild strains of *Oryza perennis* and *Oryza punctata* showed lower activities than the two cultivated species. The mean activity was 494 n moles / h /g dry root for 21 *O. perennis* strains and 205 n moles/h/g dry root for 5 *O. punctata* strains. The results indicated that the association between rice plants and N_2-fixing bacteria was controlled by the genotypes of the plants, and the association might have been enhanced in the course of domestication. After the work of Hirota *et al.*, (1978) and Sano *et al.*, (1981), the correlation between ARA and root dry weight was further tested by Ladha *et al.*, (1986). They observed significant difference in ARA among the varieties with and without straw incorporation at several stages of rice growth during both seasons (dry and wet) and the differences in ARA were also dependent on the quantity of root.

Recently in India, Ravichandran and Arunachalam (1997) observed the variation of acetylene reduction activity in the rhizosphere of different cultivated rice. The rhizosphere of IR-42 showed the maximum nitrogenase activity followed by that of IR-20, IR-8, IR-26 and IR-22. They also observed that nitrogenase activity increased with the increase in plant population. Rao

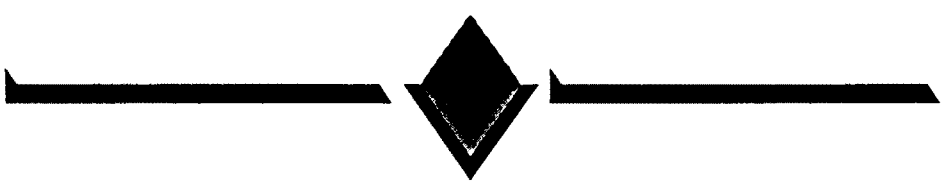
and Rao (1986) also reported that rhizosphere nitrogenase activity of 20 rice varieties differed significantly between the varieties and between sampling dates.

Dommergues (1978) reported mutants that stimulated more biological nitrogen fixation (Nfs character). In a pot experiment, N - balances of six variety showed that the correlation coefficient between nitrogen balance and nitrogen uptake for experiment 1 to 6 were 0.78, 0.68, 0.71, 0.88 and 0.76 (App *et al.*, 1986). In addition, N - balance and total dry weight also correlated, correlation of coefficient for experiment 1 to 6 were 0.65, 0.76, 0.52, 0.84, 0.59 and 0.74. Hence, it appears that a high plant biomass is associated with a positive N-balance. This may reflect the derepression of soil biological nitrogen fixation through depletion of available N by a vigorous root system. Similarly, other plant traits associated ($P < 0.01$) with associative biological N_2-fixation , determined by Ladha *et al.*, (1988), were (in order of decreasing importance) dry weight of roots and submerged portions of the plant at heading, dry weight of shoots at heading, and N -uptake at heading, and N -uptake at maturity. With the use of plant traits and a short term ARA assay, they established a ranking for biological N_2-fixation and N- utilization of 21 rice genotypes that was fairly reproducible in two consecutive dry season trials.

Recently, in International Rice Research Institute (IRRI), molecular marker facilitated investigation was done to study the genetic nature of varietal ability to stimulate N_2-fixation in rice rhizosphere (Wu *et al.*, 1995). An F_2 population, consisting of 231 individuals derived from a cross between rice cultivars with a similar growth period (Palawan and IR - 42) was utilized for the study. To assess rhizospheric N_2-fixation , an isotope enriched ^{15}N dilation technique was employed. The variety IR - 42, an indica variety, had 23% higher N derived from fixation (Ndfa) than Palawan, a javanica

genotype. Normal segregation of atom % ^{15}N excess was obtained in the F_2 population, with an average of 0.248 with 8% of plants below IR-45 (0.188) and 10% of plants above Palawan (0.248). Some 104 RFLP markers mapped on 12 chromosomes were tested for linkage to the putative QTLs. Significant ($P < 0.01$) associations between markers and segregation of atom % ^{15}N excess were observed for seven marker loci on chromosomes 1, 3, 6 and 11. Four QTLS defined by the detected marker loci were identified by interval mapping analysis. The results of this study suggest that rice genetic factors that affect levels of atom % ^{15}N excess in the soil by interacting with diazotrophs in the rice rhizosphere can be identified.

CHAPTER - III



MATERIALS AND METHODS

MATERIALS AND METHOD S

3.1. Materials

3.1.1. Soils

The detail physical and chemical characteristics of the soil used in different pot and field experiments, conducted in New Alluvial, Old Alluvial, Red Laterite, Coastal Saline, Terai and Hill zones is presented in Table No.1.

3.1.2 Rice cultivars

The following eight rice cultivars were used in various pot and field experiments. The yield potentiality, duration of the crop and prominent morphological features of each cultivars are presented below.

IR-50 : It is a derivative of the multicross IR-2153-14-1-6-2 x 12.28 x 12-36. The average plant height is 70 to 75 cm. It contains profuse productive tillers. 1000 grain weight is 20.35g. It takes 110 to 115 days to mature and is classified as a short duration variety. The average yield of this variety is 4.0 to 4.5 tonnes per hectare.

IR-64 : The variety has been derived from the cross IR5657-35-2-1 x IR-2061465-1-5-5 made at Rajendranagar, Hyderabad. The plants are erect and semi-dwarf with an average plant height of 100 cm. The grains are long slender with straw coloured husk. It is a fertilizer responsive variety suitable for both kharif and rabi season. The average yield of the variety is 5 to 6 tonnes per hectare and it takes 120 to125 days to mature.

IR-36 : This variety is highly fertilizer responsive. It can grow in medium land situation. Grains are long slender. It takes 115 to120 days to mature and gives yield upto 5 to 6 tonnes per hectare.

ıble 1 : Physical and chemical characteristics of the soils used location.

Soil	N.B. campus farm, BCKV, Cooch Behar	RRS - Kalimpong Farm, BCKV, Kalimpong, Darjeeling	RRS - Majhian Farm, BCKV, Majhian, D. Dinajpur	RRS - Chakdah Farm, BCKV, Chakdah , Nadia	RRS - Sekhampur Farm, BCKV, Sekhampur, Birbhum	RRS- Kakdwip Farm, BCKV, Kakdwip, S. 24-Parganas
Type of Soil	Terai soil	Hill soil	Old Alluvial soil	New Alluvial soil	Red and Laterile soil	Coastal saline soil
PH (1:2.5)	5.8	5.0	6.2	6.7	5.3	7.1
EC (dsm^{-1})	0.3	0.1	0.4	0.3	0.25	1.95
Org. C (gkg^{-1})	10.5	12.4	7.5	12.1	7.6	5.2
CEC (Cmol kg^{-1})	7.46	6.72	18.2	29.0	7.23	23.12
Total N (%)	0.087	0.063	0.077	0.110	0.073	0.051
Available P ($mgkg^{-1}$)	12.5	11.2	17.8	15.6	14.3	14.6
Available K ($mgkg^{-1}$)	96.9	51.3	120.5	150.7	47.3	198.5
Sand (%)	52.3	57.1	65.7	2.0	63.5	36.6
Silt (%)	40.7	37.7	15.8	46.9	19.2	40.0
Clay (%)	7.0	5.2	17.5	51.1	17.3	23.4
Textural class	Sandy loam	Sandy loam	Sandy loam	Silty clay	Sandy loam	Clay loam
Water holding capacity	41	45	40	50	42	47

BCKV - Bidhan Chandra Krishi Viswavidyalaya ; RRS - Regional Research Station.

IET-4094 : It is derived from a cross between Bu1 x CR - 115. This variety is also fertilizer responsive variety which takes 115 to 120 days to mature. Grains are long slender. This variety can grow in both up and medium land situation. The average yield of this variety is 4 to 5 tonnes per hectare.

IET-13483 :This variety is suitable for hill region, collected from Rajendranagar, Hyderabad. Average height of the plant is 100 to 105 cm. It takes 145 to 150 days to mature and gives average yield of 3.5 to 4.0 tonnes per hectare.

MW-10 : It is a selection from the cross MUT15 x YAIKYAKU NANTOKU (China). It is a semi-tall variety with an average plant height of 110.5 cm. with stiff straw. The grains are short bold having 1000 grains weight of 22.2g. The variety takes 110-115 days to mature and average yield is 3.0 to 3.5 tonnes per hectare.

CR-544 -1-1 : This variety is released from CRRI, Cuttack. It is an extra early variety and takes 78 to 80 days to mature. Grains of this variety are medium slender. It gives yield upto 3 to 4 tonnes per hectare.

Manas Sorobar : This variety is selected for lowland situation. Average plant height is 100 cm. Grains are long slender. It takes 140 to 145 days to mature and gives yield upto 4 to 5 tonnes per hectare.

3.1.3 Bacterial strains

Following sixteen diazotrophic strains were exploited in different pot and field experiment.

P3 : Gram negative, ovoid shaped, microaerophilic nitrogen fixer, isolated from paddy field of Cooch Behar, West Bengal , fix 11.12 mg atmospheric N g^{-1} of carbon source.

P4 : Gram negative, rod shaped, microaerophilic, isolated from paddy field of Cooch Behar fix 7.17 mg N g^{-1} of carbon source.

P12 : Gram negative short rod, microaerophilic nitrogen fixer, isolated from lowland paddy field of Cooch Behar. Fix atmospheric N @ 2.32 mg g^{-1} of carbon source and produce indole acetic acid @ 12.5 mg. ml^{-1} of medium.

A6 : Gram negative, rod with rounded end isolated from paddy field of Cooch Behar, fix 6.59 mg atmospheric N g^{-1} of carbon source and produce 3.92 mg IAA ml^{-1} of medium.

A7 : Gram negative, rod with rounded end, aerobic nitrogen fixer, isolated from paddy field of Cooch Behar, fix 6.92 mg molecular N g^{-1} of carbon source and produce 9.64 mg IAA ml^{-1} of growth medium.

A9 : Gram negative ovoid shaped aerobic nitrogen fixer isolated from paddy field of Cooch Behar, fix 4.13 mg N g-1 of carbon source.

A11 : Gram negative rod shaped aerobic nitrogen fixer, isolated from paddy field of Cooch Behar, fix 3.84 mg N g^{-1} of carbon source and produce 2.3 mg IAA ml^{-1} of growth medium.

A13 : Gram negative aerobic , ovoid to rod shaped, isolated from paddy field of Cooch Behar, fix 4.63 mg N g^{-1} of carbon source and produce 1.01 mg IAA ml^{-1} of growth medium.

A15 : Gram negative, ovoid to rod shaped, aerobic nitrogen fixer, isolated from paddy field of Cooch Behar, fix 4.28 mg atmospheric N g^{-1} of carbon source.

Ap3 : Gram negative, aerobic rod, microaerophilic nitrogen fixer, isolated from lowland paddy field of Cooch Behar, fix 3.29 mg atmospheric N g^{-1} of carbon source and produce 8.44 mg IAA ml^{-1} of growth medium.

Ap18 : Gram negative, curved rod, microaerophilic nitrogen fixer isolated from lowland paddy field of Cooch Behar, fix 3.95 mg N g^{-1} of carbon source.

D2: Gram negative, rod to ovoid shaped, aerobic nitrogen fixer isolated from paddy field of Cooch Behar, fix 10.08 mg N g^{-1} of carbon source.

D5 : Gram negative rod to ovoid shaped aerobic nitrogen fixer isolated from paddy field of Cooch Behar, fix atmospheric N @ 9.54 mg. g^{-1} of carbon source.

MP251 : *Azospirillum* sp. isolated from lowland paddy field of Midnapur. district of West Bengal.

3.1.4. Fertilizers and other chemicals

Urea, single super phosphate (SSP) and muriate of potash used as source of nitrogen, phosphorus and potassium, respectively, were procured from standard fertilizer company. All other chemicals used for this investigation , were obtained from reputed chemical companies.

3.2.Method

3.2.1. Isolation, purification and maintenance of diazotrophs from rice root and rhizosphere.

a) Collection of rhizosphere soil and rice root sample

Soil and root samples were collected from BCKV- farm, Pundibari (Cooch Behar Block-II), District Seed Farm, Cooch Behar and farmer's field (Cooch Behar Block-I) of Cooch Behar district during wet season, 1995. rhizosphere soil and root of rice plants were collected at heading stage from three rice varieties - Jaldhapa (local), CR-544 -1-1 and MW-10.

b) Isolation of diazotrophs from rhizosphere soil : The standard isolation procedure was followed for isolation of organisms from rhizosphere soils. For this, diluted soil samples were directly plated on the media and after 72hrs. incubation at 30°c , various representative colonies were detected for further studies. *Azotobacter* - like organisms designated as 'A' were isolated purified and maintained in Jensen N- free medium (Jensen, 1951).

Composition of the medium : Sucrose, 20.0g ; K_2HPO_4, 1.0g ; $MgSO_4$.$7H_20$, 0.5g ; $FeSO_4$, 0.1g; $CaCO_3$, 2.0g ; Agar, 15.0g ; Distilled water, 1000 ml. pH-7.0.

For *Bacillus* spp. , Hino and Wilson (1958) medium was used for isolation, purification and maintenance of the isolate marked as 'B'.

Composition of the medium : Sucrose, 20.0g ; $MgSO_4$. $7H_2O$, 0.5g ; NaCl , 0.01g ; $FeSO_4$. $7H_2O$, 0.015g ; Na_2MoO_4 . $2H_2O$, 0.005g ; $CaCO_3$, 10.0g ; *P*-amino benzoic acid, 10 µg. Biotin, 5µg ; KH_2PO_4, 0.13g ; K_2HPO_4, 0.17g ; Agar, 15.0g ; Distilled water, 1000 ml. pH 7.0.

Nitrogen free mineral glucose agar medium was used for the isolation of putative *Derxia* sp. and the isolates were designated as 'D'.

Composition of the medium : Glucose, 20.0g; K_2HPO_4 ,0.8g ;KH_2PO_4, 0.2g ; $FeCl_3.6H_2O$, 0.025g ; Na_2MoO_4. $2H_20$, 0.005g, $CaCl_2$, 0.05g ; Agar, 15.0g., Distilled water, 1000 ml. pH 6.9.

A medium described by Hill (1976) was used for the isolation, purification and maintenance of *Klebsiella* isolates, mentioned as 'K'.

Composition of medium : Sucrose, 20.0g ; Na_2HPO_4, 10.4g ; KH_2PO_4, 3.4g ; Iron (III) citrate, 36.0 mg ; $MgSO_4$, 0.3mg ; $CaCl_2.2H_2O$, 26.0mg ; $MnSO_4$, 0.3mg ; $Na_2MoO_4.2H_2O$, 7.6mg ; Agar, 15.0g ; Distilled water, 1000 ml. The phosphates were sterilized separately and added to the bulk of the medium when cool.

c) Isolation of diazotrophs from rice roots

Isolation procedures from rice root followed was essentially the same as followed by Watanabe and Barraquio (1979) at International Rice Research Institute, for the study of endorhizospheric micro-organisms in wetland rice. The washed roots were cut into segments and macerated to provide a suspension of bacteria. The diluted sample was inoculated into semisolid

media for proper enrichment and subsequently streaked on solid media for isolation.

Modified Dobereiner medium (You and Zhou, 1989) was used for enrichment and isolation of *Alkaligenes* - like bacteria, all these isolates were designated as 'Al'.

Composition of the medium : Sodium lactate (70%), 7.5 ml ; Yeast extract, 0.1 g ; KH_2PO_4, 0.4 g ; K_2HPO_4, 0.1 g ; $MgSO_4.7H_2O$, 0.2 g ; NaCl, 0.01g ; $Fe_2(SO_4)_3.H_2O$, 0.01g ; $MnSO_4 .H_2O$, 0.01g ; $MnSO_4 .7H_2O$, 0.01g ; $Na_2MoO_4 .2H_2O$, 0.01g ; Agar, 1.75g (for semisolid) or 15.0g (for solid) ; Distilled water, 1000 ml.

For enrichment of *Pseudomonas* - like bacteria (P), semisolid glucose yeast extract medium (Watanabe and Barraquio, 1979) was used and the same solid medium was used for purification and maintenance of the bacteria.

Composition of the medium : Glucose, 5.0g ; Yeast extract, 0.1g ; K_2HPO_4, 0.5g ; $FeSO_4.7H_2O$, 0.04g ; $MgSO_4.7H_2O$,0.2g ; $Na_2MoO_4.2H_2O$, 0.005g ; $CaCl_2.2H_2O$, 0.2g ; H_3BO_3, 0.15 mg ; $ZnSO_4.7H_2O$, 0.11 mg ; $CoSO_4 .7H_2O$, 0.7 mg ; $CuSO_4.7H_2O$, 0.005 mg ; $MnCl_2.4H_2O$, 0.004 mg ; Distilled water, 1000 ml. Agar, 1.75g ; pH 7.0.

Azospirillum, designated as 'Ap', were enriched in semisolid nitrogen free malate medium (Dobereiner, 1980) and subsequently streaked on the same solid medium supplemented with 0.02g yeast extract / litre - where it appeared as typical small, white dense single colony after one week of incubation at 30°c. The cultures were maintained in semisolid medium .

Composition of the medium : Malic acid , 5.0g ; K_2HPO_4, 0.5g ; $MgSO_4.7H_2O$, 0.2g ; NaCl, 0.1g ; $CaCl_2$, 0.02g ; $Na_2MoO_4.2H_2O$, 0.002g ; $MnSO_4.7H_2O$, 0.01g ; Fe-EDTA (1.64% W/V, aqueous), 4.0 ml ;

Bromothymol Blue (0.5% w/v in ethanol), 3.0 ml; KOH, 4.5g; Biotin, 0.001g ; Agar, 1.75g ; Distilled water, 1000 ml ; pH 6.8 - 7.0.

3.2.2 Screening of diazotrophs

All the isolates were tested for their capacity to fix atmospheric nitrogen with the help of ARA and Kjeldahl method. Isolates were also examined for IAA production capacity.

3.2.2.1 Measurement of Acetylene Reduction Activity (ARA)

All the isolates were tested for acetylene reduction activity in various media with different incubation period. Isolates designated as 'A' and 'D' were grown in same solid media which were used for their isolation. Similarly, other isolates were grown in their semisolid isolation media except the culture 'B' and 'K' which were grown in Rennie's Combined Carbon Media (semisolid).

Cultures were grown in 10ml glass vials having 5ml medium in each and were allowed to incubate. Isolate P4 was grown for 3 days, P12 for 5 days and other were grown for 2 days at 30 °C. After the required growth of each culture, cotton plugs were replaced with rubber serum caps, then 10% air was withdrawn and same volume of C_2H_2 was introduced. Before the assay of ethylene, *Azotobacter* and *Derxia* like bacteria were incubated for one hour whereas, others were incubated for 24 hrs at 30°C. The amount of C_2H_4 formed was measured with a gas chromatograph (HP, model- 5730A) fitted with a glass column containing porapak R (80 - 100 mesh) and equipped with a flame ionization detector. The oven temperature and carrier gas (N_2) flow rate were 80 °C and 80 ml/minute, respectively.

Composition of Combined Carbon Medium (Rennie, 1981) :

Solution A ; K_2HPO_4, 0.8 g ; K_2HPO_4, 0.2 g ; NaCl, 0.1 g ; Na_2 Fe-EDTA , 0.028 g ; Na_2MoO_4 . $2H_2O$, 0.025 g ; Yeast extract, 0.1 g ; Mannitol, 5.0 g ; Sucrose, 5.0 g ; Na-lactate (60% w/v), 0.5 ml ; Agar, 15.0 g ; Distilled water, 9000 ml.

Solution B : $MgSO_4$.$7H_2O$, 0.2 g ; $CaCl_2$, 0.06 ; Distilled water, 100 ml.

The solutions were autoclaved separately, cooled and mixed. Filter sterilized biotin (5µg /litre) and *P*-amino benzoic acid (10 µg /litre) were added and the final pH was adjusted to 7.0.

3.2.2.2 Estimation of nitrogen fixing capacity of diazotrophs by Kjeldahl method

All the purified culture were tested for N_2 -fixing capacity by Kjeldahl method. Cultures were inoculated into respective isolation media in three replicates. Cultures designated 'A' and 'D' were inoculated into 25 ml liquid medium, whereas, Cultures designated 'A' and 'P' were tested in 50ml semisolid media. Appropriate uninoculated controls were also maintained for each culture. The inoculated flasks were then incubated at 30°C for two weeks. N -fixed was estimated by Kjeldahl method of digestion and distillation in Bremner's apparatus. Results were expressed as mg N fixed per g of sugar consumed considering the quantity of respective sugar present in that particular medium.

3.2.3.3 Colorimetric quantification of IAA production by diazotrophs

For detection and quantification of indole acetic acid (IAA) production by diazotrophic bacteria, cultures were grown in Okon's malate medium (Okon *et al.*, 1977) enriched with Sucrose, 5.0 g ; mannitol, 5.0 g and Ammonium chloride, 1.0 g per litre of medium. Tryptophan @ 100 mg per

litre was added as precursor of indole acetic acid. One loopful of culture was inoculated in 25ml broth and after incubation for two weeks at 30°c the culture was centrifuged at 1000 rpm. for 15 minutes. 2 to 3 drops of o-phosphoric acid and 4.0 ml of reagent (1ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) was added to 2.0 ml of supernatant, and after 25minutes at room temperature, absorbance was recorded at 530 nm. To quantify the IAA production, absorbance was compared with calibration curve made by using IAA as standard (0.5 to 50 µg / ml).

3.2.3. Characterization of bacterial isolates

3.2.3.1. Morphological studies of bacterial isolates

Shape and size of bacteria :

Young (48 hours growth) cultures were used to study the shape and size of the bacteria. Bacterial smear was stained with nigrosine (Negative staining) and observed using oil immersion lens of a light microscope. Length/diameter of bacterial cells was measured with ocular micrometer.

Composition of nigrosine solution : Nigrosine 10.0g ; Distilled water, 100ml. After boiling for 30 minutes, 0.5 ml formaldehyde (40%) was added and filtered twice through filter paper.

Motility and cellular arrangement :

Broth culture of 48 hours growth was used to determine cellular arrangement and motility of bacteria. Motility of the bacteria was examined by hanging drop method.

Gram staining :

Young culture was used for gram staining. Air dried smear was prepared and passed several times through the top of a bunsen flame for heat

fixation. The smear was stained with crystal violet solution (A) for 1 minute, rinsed lightly with water and excess water was drained off. After rinsing with water, it was flooded with iodine solution (B) and allowed to act for 1 minute. Iodine solution was drained off and decolourized with iodinated alcohol (C) until all free blue colour had been removed. After washing the smear with distilled water, counter staining was done with safranin (D) for 1 minute. Slide was then washed with distilled water, dried and observed under oil immersion objective.

Composition of Gram's Reagent :

A. Crystal violet solution : Crystal violet, 10.0 g ; Ammonium oxalate, 4.0 g; Ethanol, 100 ml ; Distilled water, 400 ml.

B. Iodine solution : Potassium iodide, 2.0 g ; Distilled water, 300 ml ; Iodine, 1.0 g.

C. Alcohol (iodinated) : Iodine solution (B), 5.0 ml ; Ethanol, 95.0 ml.

D. Safranin solution : Safranin, 0.25 g ; Ethanol, 10 ml ; Distilled water, 90 ml.

Spore staining :

For spore staining heat fixed smear was covered with malachite green (5.0 g malachite green oxalate dissolved in 100 ml distilled water) and steamed over boiling water for 5 minutes. The slide was cooled and rinsed with water for 30 seconds. Counter staining was done with safranin (Gram's) for about 30 seconds, rinsed with water to remove safranin, blot dried and examined under oil immersion objective.

Capsule staining :

For capsule staining air dried smear of 48 hours culture was stained with crystal violet solution (10 g crystal violet in 100 ml distilled water) for one minute. Crystal violet stain was then washed with 20% $CuSO_4$ solution.

Bolt dried slide was observed under oil immersion lens and capsule was detected as light blue or colourless halo around the bacterial cell.

3.2.3.2 Cultural Characteristics

Colony characters :

To study the colony characters the same media were used that were previously used for isolation of diazotrophic bacteria. In addition to that BMS-agar, solid nitrogen free Na-malate medium (Dobereiner, 1980) supplemented with 0.02 g yeast extract/litre, and yeast extract mannitol agar (Barraquio and Watanabe, 1981) were also used to study the colony characteristics.

Composition of BMS-agar : Washed, Peeled, sliced potatoes ; 200 g ; L-malic acid, 2.5 g ; KOH, 2.0 g ; Raw cane sugar, 2.5 g ; Vitamin solution (Biotin, 0.01 g ; Pyridoxin, 0.02 g ; Distilled water, 1000 ml), 1.0 ml ; Bromothymol blue (0.5 % alcoholic solution), 2 drops ; Agar, 15.0 g. Potatoes were placed in a guage bag, boiled in 1 litre of water for 30 minutes, then filtered through cotton, filtrate was used and volume was made upto 1 litre.

Pigment production

For pigment production, cultures were grown on iron deficient medium in the presence and absence of benzoate. Medium of Norris and Jensen (1958) was modified by omission of $FeSO_4$ $7H_2O$ and reduction of Na_2MoO_4 $.2H_2O$ to 1µg/ml (Thompson and Skerman, 1979). Glucose or sodium benzoate was added to give a concentration of 10 g/litre. The medium was inoculated by depositing a small drop of a suspension of culture on the surface of the agar. The plates were examined in daylight for diffusible and non diffusible pigment and under ultraviolet light for fluorescent pigment.

Composition of Norris and Jensen medium (1958) (g/litre) : K_2HPO_4, 1.0 ; $CaCl_2$. $2H_2O$, 0.1 ; $MgSO_4$.$7H_2O$, 0.2 ; $FeSO_4$.$7H_2O$, 0.05 ; $Na_2MoO_4.2H_2O$, 0.005 ; Agar, 15.0 ; pH 7.3.

3.2.3.3 Physiological Characteristics

Oxidase test (Kovacs, 1956)

For oxidase test, culture was grown for 24 - 48 hours. Colony was picked up with a clean sterile glass rod and smeared on the wet oxidase disc (soaked with 1.0% tetramethyl - *p* - phenylene - diamine HCl in distilled water). A positive reaction was indicated by a change in color within 5 - 10 seconds as appearance of deep purple blue. A delayed positive reaction appeared in 10 - 60 seconds while a change later than 60 seconds or no change at all was considered negative reaction.

Catalase test (Taylor *et al.*, 1972)

An agar slant of respective culture medium was inoculated heavily with the test bacterial isolate. After incubation at an optimal temperature for 24 - 48 hrs, 1.0 ml of 3.0% hydrogen peroxide was introduced in tubes, allowing it to flow over the slant. Positive test for the enzyme was indicated by the production of gas bubbles.

Methyl red test (Clark and Lubs, 1915)

MR-VP broth was dispensed into tubes and sterilized by steaming for 30 minutes for 3 successive days. After inoculation, tubes were incubated at 35°C for 5 days. About 5-6 drops of methyl red reagent was added to each tube and formation of bright red colour was indicated as positive result. Formation of yellow or orange colour was considered as negative reaction.

Composition of MR-VP broth (Clark - Lubs broth) : Protease peptone, 5.0 g ; Glucose, 5.0 g ; NaCl, 5.0 g ; K_2HPO_4, 5.0 g ; Distilled water, 1000 ml ; pH 7.0.

Methyl red reagent : 0.1 g methyl red was dissolved in 300 ml of 95% ethanol and volume was made with distilled water upto 500 ml.

Voges Proskauer test (Burrit, 1936)

MR-VP broth was inoculated with bacterial isolates and incubated at 35°C for 48 hours. After incubation, 0.6 ml of 5% naphthol (in 95% alcohol) and 0.2 ml of 40% aqueous solution of KOH were added to 1.0 ml of culture broth and shaken well. Observation was taken after one hour, development of red or pink colour was noted as positive test.

Hydrolysis of Starch

For starch hydrolysis test, procedure followed was that described by Blazevic & Ederer (1975). Starch agar plates (Nutrient agar +1% soluble starch) were inoculated and incubated at 35°C for 24-48 hours. After incubation, plates were flooded with grams iodine solution. Hydrolysed starch appeared as colourless, transparent zone around the colony against blue colour.

Hydrolysis of Casein

For casein hydrolysis test, procedure followed was that of Harry and Paul's (1962). Skim milk agar plates (Nutrient agar + 10% Skim milk) were spot inoculated and incubated at optimum temperature for 2-3 days. Clear zone around the colony was noted as positive test for casein hydrolysis.

Hydrolysis of gelatin (Frazier, 1926)

Frazier's gelatin agar plates (Nutrient agar + 0.4% gelatin) was spot inoculated and incubated at 30°C for 3 days. After incubation, mercuric chloride solution was flooded on the plates. Hydrolysed gelatin appeared as clear zone around the colony, where as, unhydrolysed gelatin formed white precipitate with the reagent.

Composition of mercuric chloride : $HgCl_2$, 15.0 g ; Conc. HCl, 20.0 ml, Distilled water, 100 ml.

Hydrolysis of urea (Christensen, 1946)

Christensen urea agar plates were inoculated and incubated at 35°C upto 4 days. Production of urease was indicated by the change of colour from yellow to red or pink, as a result of increase in pH of the medium.

Composition of urea agar :

(a) **Agar base** : Agar, 15.0 g ; Distilled water 900 ml, autoclaved at 121°C for 20 minutes.

(b) **Urea base** : Peptone, 1.0 g ; Glucose, 1.0 g ; KH_2PO_4, 2.0 g ; NaCl, 5.0 g; Urea, 20.0 g ; Phenol red, 0.012 g ; Distilled water, 100 ml ; pH 6.8-7.0.

Nitrate reduction test (Blazevic *et al.*, 1973)

Nitrate broth (Peptone water + 1.0 g KNO_3 per litre)was prepared and dispensed in tubes with inverted Durham tube. Tubes were inoculated and incubated at 35°C for 48 hours. After incubation, 0.5 ml each of sulphanilic acid (sulphanilic acid, 0.8g in 100 ml 5 N acetic acid) and dimethyl -α-naphthylamine (0.5% in 5 N acetic acid) were added to each culture tube. The conversion of NO_3 to NO_2 was indicated as red colour. To confirm the presence of nitrate in the negative reaction a bit of zinc dust was added to the medium (zinc reduces the nitrate in the medium). Presence of gas bubble in the Durham tube was considered as gas production from nitrate in case of nonfermentative organisms.

Indole test (Kovacs, 1928)

Tryptophan broth tubes were inoculated with bacterial isolates and incubated at 35°C for 2-3 days. Indole production was tested by adding 0.5 ml Kovacs reagent. Red colour in the surface layer indicated positive result.

Composition of Tryptophan broth :

Tryptone, 10.0g ; NaCl, 5.0g ; Distilled water, 1000 ml; pH 7.0.

Composition of Kovacs reagent :

P - dimethylaminobenzaldehyde, 10.0g ; *iso* - amyl alcohol, 150 ml ; conc. HCl, 50.0 ml.

Test for citrate utilization (Simmons, 1926)

Simmon's citrate medium containing bromothymol blue was prepared, dispensed into tubes, autoclaved at 121°C for 20 minutes and slants were prepared. After inoculation, all slants were incubated at 35°C for 24-48 hours. Positive results were noted by observing the growth and change of colour from green to blue.

Composition of Simmon's Citrate medium (g/l) :

$MgSO_4.7H_2O$, 0.2 ; $(NH_4)\ H_2PO_4$, 1.0 ; K_2HPO_4, 1.0 ; NaCl, 5.0 ; Na-Citrate, 2.0 ; Bromothymol blue, 0.08 ; Agar, 15.0 ; Distilled water, 1000 ml ; pH 6.8-7.0.

Test for organic acid utilization

For organic acid utilization test, Sodium salts of lactic acid, fumaric acid , malic acid, succinic acid, acetic acid, and propionic acid were used.

Organic acid was added to Kosar's basal medium (modified by adding 2 ml of 0.5% alcoholic solution of bromothymol blue) to give 1.0% concentration. After autoclaving at 121°C for 20 minutes, medium was poured into petriplates and allowed to solidify. Spot inoculation was done with isolates and incubated at 35°C for 2-3 days. Alkaline reaction was noted as positive result.

Kosar's basal medium (Kosar, 1923) (g/l) : NaCl, 5.0 ; $MgSO_4.\ 7H_2O$, 0.2 ; $(NH_4)\ H_2PO_4$, 1.0 ; K_2HPO_4, 1.0 ; Agar, 15.0 ; Distilled water, 1000 ml ; pH 6.8.

Test for utilization of simple sugars and sugar alcohols as sole Carbon Sources :

All the bacterial isolates were tested for their ability to utilize glucose, galactose, fructose, arabinose, sucrose, maltose, lactose, *m*-inositol, glycerol and mannitol as sole carbon source.

For this test basal medium was sterilized by autoclaving at 121°C for 20 minutes. Filter sterilized carbohydrate solution was added to each flask (at 45°c) to give a final concentration of 1.0% and the medium was plated. Each plate was spot inoculated by two isolates at a time and incubated for 3 - 4 days at 35°c. Any visible growth was recorded as positive test.

Composition of basal medium (g/l) : $(NH_4)_2SO_4$, 1.0; $MgSO_4$. $7H_2O$, 1.0 ; K_2HPO_4, 2.0 ; $FeCl_3$ $6H_2O$, 0.0047 ; $MgSO_4$. H_2O, 0.0025 ; $ZnSO_4$. $7H_2O$, 0.00072 ; $CuSO_4$. $5H_2O$, 0.000125 ; $CoSO_4$. $7H_2O$, 0.00014 ; H_3BO_3, 0.000031 ; Na_2MoO_4. $2H_2O$, 0.000245 ; Biotin, 0.0001 ; $CaCO_3$, 0.001 ; Agar, 16.0 ; Distilled water, 1000 ml ; pH 7.0.

3.2.4 Pot Experiment

3.2.4.1. Screening of effective "Diazotroph x Rice cultivar" combination.

Location : The pot experiment was conducted in a net house at North Bengal Campus, BCKV, Cooch Behar.

Soil : Soil was collected from BCKV - farm, North Bengal Campus, Cooch Behar. Physico-chemical properties of soil are presented in Table - 1.

Rice cultivar : Three rice cultivars, CR - 544 - 1 - 1, MW - 10 and IR - 50 were used for the present study.

Season : Wet season, 1996.

Bacterial strains : Bacterial isolates marked as A6, A7, Ap3, Ap18, D2, P3, P4, P12, were used for inoculation in the experiment. All the eight strains were isolated from the wetland paddy field of Terai zone.

Experimental Details : Soil was passed through a 2 mm sieve and mixed to homogeneity. Each of the earthen pots (28 cm diameter and 25 cm height) lined with polyethylene bag was filled with 8.0 kg of above soil. Each pot was fertilized with N, P_2O_5, K_2O and ammonium molybdate @ 20 kg, 40 kg, 40 kg and 0.2 kg ha^{-1}, respectively. Manuring was done @ 5000 kg FYM ha^{-1}. Nitrogen was applied two weeks after transplantation.

Each rice cultivar was inoculated with each of the eight different isolates. Control was maintained for each variety inoculated with heat killed bacteria. Six pots were used for each treatment. Seedlings of 20 days old were transplanted after inoculation. Two plants per pot were maintained in each treatment.

Inoculum preparation and inoculation :

All the eight cultures, selected for inoculation were grown in Rennie's combined carbon medium (Rennie, 1981) separately. The isolates from stock culture were transferred to 500 ml flasks containing 200 ml broth and after incubation the cells were centrifuged at 8000 rpm for 10 minutes and finally suspended in same combined carbon medium (CCM) at a concentration of approximately 10^8 cells / ml.

Inoculation was performed twice Roots of seedlings, freed from seed bed soil by washing with distilled water, were dipped into said bacterial suspension for 24 hrs and then inoculated rice seedlings were transplanted to pot. Appropriate control was maintained with heat killed bacteria. Subsequently 15 days after transplanting, a 5.0 ml suspension of each bacterium was inoculated with a sterile pipette into the rice plant in each pot of inoculated treatments.

After 45 days of transplantation, observations were recorded on number of tiller per pot, dry weight of shoot and total N-uptake per pot.

Enumeration of bacteria in rhizosphere and rhizoplane of rice

Rice plants were dug from the soil at maximum tillering stage and soil adhering to roots was gently removed from roots. The removed soil was used as rhizosphere soil sample. The soil was mixed thoroughly and a subsample was used immediately for enumeration of bacterial population, keeping a set of sample for moisture estimation in order to express the results on dry weight basis. For enumerating rhizoplane bacteria, roots were washed gently under running water to remove the bulk of adhering soil and then rinsed four times in sterile water. Roots were cut into 1.0 to 2.0cm segments and then blotted gently with sterile tissue paper. A 1.0 g portion of segments was placed in a 250 ml flask containing 100ml of sterile water and 5.0g of glass beads, which was shaken vigorously for 20 minutes. The bacteria dislodged by shaking were considered as rhizoplane bacteria (Watanabe *et al.,* 1979). Total aerobic nitrogen fixer, *Azotobacter, Azospirillum* and *Pseudomonas* like organisms were enumerated using combined carbon medium (Renni, 1981), Jensen medium (Jensen, 1951), semisolid Glucose yeast extract medium and Malate yeast extract medium (Watanabe and Barraquio, 1979 ; Watanabe *et al.,* 1979), respectively. *Azospirillum* and *Pseudomonas* like bacteria were enumerated by MPN method with 10 fold dilution and 5 tubes per dilution (Alexander, 1982).

3.2.4.2. Performance of carrier based Diazotrophs strains for rice var. IR-50

Location : North Bengal Campus, BCKV, Cooch Behar.

Soil : Farm soil of North Bengal Campus, BCKV, Cooch Behar.

Rice variety and Bacterial Culture :

Rice variety IR-50 and charcoal based inoculum of isolates-A6, A7, Ap3, Ap18, D2, P3, P4, and P12, were used in the experiment.

Season : Kharif Season, 1996.

Experimental Details :

The experiment was conducted with 9 treatments and 6 replications. About 8.0 kg soil was used for each pot (28cm diameter and 25cm height). Potting of soil was done as described in case of previous pot experiment. Each pot received N, P_2O_5, K_2O and FYM @ 50 kg, 50 kg, 50 kg and 5 tha^{-1}. Phosphorus, potash and FYM were applied before transplantation, whereas N was applied into three equal splits. Seedlings (25 days old) were inoculated by seedling dipping in carrier based slurry of inoculum before transplantation and two hills per pot were maintained in each treatment. Observations on various growth parameters and yield attributes were taken at harvest.

3.2.4.3. Performance of mixed inocula over single inoculum on rice

Location : North Bengal Campus, BCKV, Cooch Behar.

Soil : Farm soil of North Bengal Campus, BCKV, Cooch Behar.

Rice variety and Bacterial Culture

Rice variety IR-50 and charcoal based inoculum of isolates-A6, Ap18, D2, P3, P12, were used in the experiment.

Experimental Details :

The experiment was designed with 10 treatments and 6 replications. Roots of rice seedlings were dipped in the slurry of carrier based inoculum of A7, Ap18, D2, P3, P12, and mixed inocula of Ap18 + A6, Ap18 + D2, Ap18 + P3, Ap18 + P12. Uninoculated control was also maintained. Each pot (containing 8.0 kg soil) was amended with N, P_2O_5, K_2O and FYM @ 50 kg, 50 kg, 50 kg and 5 tha^{-1}, respectively. Phosphorus, potash and FYM applied as basal, whereas, N was applied into three splits. Two hills per pot were maintained in each pot. Count on diazotrophic bacteria was performed at

tillering stage and the methods described in previous experiment were used for enumeration. Observations on various growth parameters were recorded at harvest.

3.2.5. Field Trial

3.2.5.1 Experiment on method of inoculation :

Location	: Chakdah, RRS, New Alluvial Zone.
Season	: Kharif, 1996.
Treatments	: T_0 - Uninoculated control ;
	T_1 - Seedbed inoculation ;
	T_2 - Seed inoculation ;
	T_3 - Seedling inoculation.
Number of replications	: Three
Design	: RBD
Plot size (mt^2)	: 3.0 x 2.0
Rice variety used	: Manas Sorobar.
Inoculant used	: Charcoal based inoculum of A13 + D18
Fertilizers used	: N, P_2O_5 & K_2O @ 80kg, 40kg & 40 kg ha^{-1}, respectively.
Manure used	: FYM @ 10 t ha^{-1}
Age of seedlings	: 35 days.
First top dressing	: 3 WAT
Second top dressing	: 6 WAT
Weeding	: once
Irrigation	: once.
Plant protection measure	: Not applied.

3.2.5. Comparative performance of different diazotrophic bacterial strains on the basis of field performance :

With the objective to compare different isolates 4 experiments were conducted at three zones of West Bengal - New Alluvial Zone (NAZ) Red and Lalterite Zone (RLZ) and Coastal Saline Zone (CSZ). The experimental details are given below.

Locations	Chakdah, RRS, NAZ	Sekhampur, RRS, RLZ	Kakdwip, RRS, CSZ
Season	Kharif ' 96 & 97 Kharif, 1996	Kharif, 1997	Kharif, 1997
Treatments (Diazotrophs)	A7, A9, A11, A15, D2 D5, D18, MP251 BU351 Kharif, 1997 : A6, A7, Ap3, Ap18, D2, P3, P4, P12 Uninoculated control.	A6 , A7 , Ap3,Ap18 D2 , P3 , P4, P12, Uninoculated control	A6 , A7 , Ap3,Ap18 D2 , P3 , P4, P12, Uninoculated control
Replications	3 (1996) ; 4 (1997)	4	4
Design	RBD	RBD	RBD
Plot size (m^2)	6.0	5.0	5.0
Rice variety	Manas Sorobar (1996) IET - 4049 (1997)	IR - 36	IR - 64
Method of inoculation	Seedling dipping	Seedling dipping	Seedling dipping
Fetilizer used	1996 : N_{60}, $P_{40}(P_2O_5)$ & K_{40} (K_2O) 1997 : N_{60}, $P_{40}(P_2O_5)$ & K_{40} (K_2O)	N_{60}, $P_{40}(P_2O_5)$& K_{40} (K_2O)	N_{60}, $P_{40}(P_2O_5)$& K_{40} (K_2O)
Manure	1996 : 10 t ha^{-1} 1997 : 2 t ha^{-1}	2 t ha^{-1}	2 t ha^{-1}
Application of N	1996 : 3 WAT, 6 WA 1997 : 2 WAT, 4 WAT & 6 WAT.	2 WAT, 4 WAT & 6 WAT	2WAT,4WAT & 6 WAT

3.2.5.3 Performance of biofertilizer under different organic sources

Location	: Chakdah, RRS, New Alluvial Zone.
Season	: KharIf, 1996.
Treatments	: Control, rabbit litter, poultry litter, sheep litter & cowdung @ 10 tha^{-1}
Number of replications	: Three
Design	: RBD
Plot size	: 3.0 m x 2.0m
Rice variety used	: Manas Sorobar.
Inoculant used	: A13 + D18.
Fertilizers used	: N_0, P_2O_5, , K_2O@ 80kg, 40kg & 40 kg ha^{-1}, respectively.
Age of the seedlings	: 35 days.
First top dressing	: 3 WAT
Second top dressing	: 6 WAT
Weeding	: once
Irrigation	: once.
Plant protection chemicals	: Not applied.

3.2.5.4 Effect of organic matter application on the effectivity of biofertilizer (N_2-fixer) :

Three experiments were conducted at two locations to assess the effect of organic manure (FYM) on the effectivity of biofertilizer. The experimental details are presented below.

Locations	Chakdah, RRS	Kakdwip, RRS Coastal
	New Alluvial Zone	Saline Zone
Season	Boro, 1997, Kharif, 1997	Boro, 1997
Treatments	FYM Levels	
	T_1 - applied once a year @ 2 t ha^{-1}	T_1 - FYM @ 5 t ha^{-1}
	T_2 - applied twice a year @ 2 t ha^{-1}	T_2 - No FYM (Control)
	T_3 - applied every alternate year @ 2 t ha^{-1}	
	T_4 - Control.	
FYM enrichment		
	Two years for Boro, 1997.	
	Two and half years for Kharif, 1997	
	Nitrogen levels	
	N_{70}, N_{80}, N_{90}, N_{100} (Boro 1997)	N_{70}, N_{80}, N_{90}, N_{100}
	N_0, N_{30}, N_{60}, N_{90} (Kharif 1997)	
Replications	3 (Boro, 1997) ; 4 (Kharif, 1997)	3
Design	Split plot(Main plot FYM	Split plot (Main plot - FYM
	sub plot - N)	sub plot - N)
Sub plot size(m^2)	6.0	2.0
Rice variety	IET-4094	IR-64
Inoculant	A11 + D2	A11 + D2
Method of inoculation	Seedling dipping	Seedling dipping
Basal fertilizer applications	N_0, P_{40} (P_2O_5), K_{40} (K_2O) in boro - 1997 N_0, P_{60} (P_2O), K_{60} (K_2O_5) in kharif , 1997 .	N_0, P_{60} (P_2O_5), K_{60} (K_2O)
Applications of N	Three splits	Three splits
	Boro, 1997 : 3 WAT	2 WAT, 5 WAT
	5 WAT & 7 WAT	7 WAT
	Kharif, 1997 : 2 WAT,4 WAT, 6 WAT	

3.2.5.5 Performance of biofertilizers under different levels of inorganic nitrogen in New Alluvial Zone (Kharif, 1996)

Location	: Chakdah, RRS, New Alluvial Zone.
Season	: Kharif, 1996.
Treatments	: N_{70}, N_{80},N_{100}, N_{70} + Inoculation ,N_{80} + Inoculation, N_{100} + Inoculation.
Number of replications	: Three
Design	: RBD
Plot size	: 3.0 m x 2.0m
Rice variety used	: Manas Sorobar.
Inoculant used	: A13 + D18.
Method of inoculation	: Seedling dipping
Fertilizers & manure used	: P_2O_5,K_2OandFYM@40kg,40kg&10 t ha^{-1}, as basal.
First top dressing	: 3 WAT
Second top dressing	: 6WAT
Weeding	: once
Irrigation	: Two.
Plant protection chemicals	: Not applied.

3.2.5.6 Performance of biofertilizers in rice under different nitrogen regimes in New Alluvial Zone , Old Alluvial, Red Laterite, Coastal Saline Zones (Kharif, 1997)

Locations	Chakdah, RRS New Alluvial	Majhian, RRS Old Alluvial	Sekhampur, RRS Red Laterite	Kakdwip,RRS Coastal Saline
Season	Kharif, 1997	Kharif, 1997	Kharif, 1997	Kharif, 1997

Treatemnt	N_0+Biofertilizer N_{30}+Biofertilizer N_{60}+Biofertilizer N_{90}+Biofertilizer	N_0+Biofertilizer N_{30}+Biofertilizer N_{60}+Biofertilizer N_{90}+Biofertilizer	N_0+Biofertilizer N_{30}+Biofertilizer N_{60}+Biofertilizer N_{90}+Biofertilizer	N_0+Biofertilizer N_{30}+Biofertilizer N_{60}+Biofertilizer N_{90}+Biofertilizer
Replication	4(Four)	4(Four)	4(Four)	4(Four)
Design	RBD	RBD	RBD	RBD
Plot Size(m^2)	6.0	6.0	5.0	4.0
Rice variety	IET-4094	IET-4094	IR-36	IR-64
Culture	A11 +D2	A11 +D2	A11 +D2	A11 +D2
Method of inoculation	Seedling dipping	Seedling dipping	Seedling dipping	Seedling dipping
Basal fertilizer application	N_0,P_{40}, K_{40} & FYM@ 2 t ha^{-1}	N_0,P_{40}, K_{40} & FYM@ 2 t ha^{-1}	N_0,P_{40}, K_{40} & FYM@ 2 t ha^{-1}	N_0,P_{40}, K_{40} &FYM@ 2 t ha^{-1}
Applicatin of N	3 splits 2,4,6 WAT	3 splits 2,4,6 WAT	3 splits 2,4,6 WAT	3 splits 2,4,6 WAT

3.2.5.7 Performance of biofertilizers under different nitrogen regimes in Terai and Hill soil (Kharif , 1997)

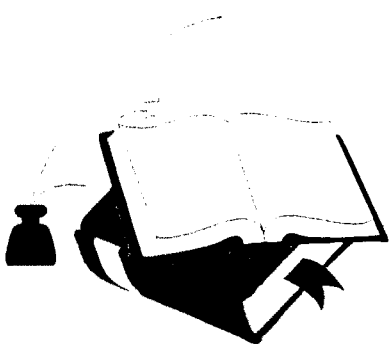
Location	Cooch Behar, RRS Terai Zone	Kalimpong, RRS Hill Zone
Season	Kharif, 1997	Kharif, 1997
Treatments	N-level , 4 (N_0, N_{30} N_{60}, N_{90}) Inoculation - 2 (with and without inoculation)	N -level , 4 (N_0, N_{20}, N_{40}, N_{60}) Inoculation - 2 (with and without inoculation)
Replication	4	4
Design	Split plot (main plot inoculation sub plot - N level)	Split plot (main plot inoculation sub plot - N level)
Sub plot size (m^2)	10	4
Rice variety	IR-50	IET-13483

Bacterial culture	Ap18 + A7	Ap18 + A7
Method of inoculation	Seedling dipping	Seedling dipping
Fertilizers & Manure	P_2O_5 - 50 $kgha^{-1}$	P_2O_5 - 40 $kgha^{-1}$
	K_2O- 50 $kgha^{-1}$	K_2O- not applied
	FYM - 5 t ha^{-1}	FYM - 10 t ha^{-1}
Application of N	$1/4^{th}$ as basal ;	$1/4^{th}$ as basal
	1/2 at tillering stage	1/2 at tillering stage
	$1/4^{th}$ at panicle	$1/4^{th}$ at panicle
	initiation stage.	initiation stage.

3.2.6. Statistical analysis of the data

The data obtained from the experiments were statistically analysed by adopting the analysis of variance technique suitable for RBD, CRD & split plot design as described by Gomez and Gomez (1984).

CHAPTER - IV



RESULT AND DISCUSSION

RESULTS AND DISCUSSION

4.1. Isolation and screening of diazotrophs

Rice root being a suitable niche for proliferation of microbes can stimulate the growth of aerobic, microaerophilic, anaerobic and faultatively anacrobic diazotrophs in its root region. With this scientific back ground, the present study has been undertaken to isolate various aerobic and microaerophilic N_2-fixers from rice rhizosphere using different specific media. Effect has also been given to screen those isolates on the basis of acetylene reduction activity (ARA), mg N fixed g^{-1} of carbon source and production of indole acetic acid (IAA).

4.1.1. Acetylene reduction activities of isolated diazotrophs

During isolation, 98 isolates were selected from various isolation media, purified and tested for ARA. Among the total isolates, only one third showed positive ARA. Predominant colonies isolated from media used for *Azotobacter, Azospirillum* and *Derxia* exhibit higher percentage of positive ARA, whereas, none of the isolates designated 'B', 'K' and 'A1' showed such positive result. ARA results indicate that 14 isolates (77.7%) of 'A', 13 (72.2%) of 'D', 3 (50.0%) of 'Ap' and 3 (25.0%) of 'P' have positive activities. In general, isolates of *Pseudomonas*-like (P) organisms scored maximum activity followed by Derxia (D), *Azotobacter* (A) and *Azospirillum* (Ap) like organisms (Table-2).

A great deal of variations in ARA were also observed among the diazotrophs isolated from same medium having similar colony characteristics. ARA of *Azotobacter* isolates varied from 6.77 to 59.9 n moles C_2H_4/vial/hr. *Azospirillum* showed very low ARA ranging from 0.52 to 3.19 n moles, but

isolates designated as 'P' exhibit very high ARA ranging from 54.62 to 412.49 n moles C_2H_4/Vial/hr.

Previous workers found the distribution of *Klebsiella* sp. (Ladha *et al.*,1983; you *et al.*, 1985) and *Alkaligenes* sp. (You *et al*, 1983; Qiu *et al* .,1981) in the rhizosphere of rice. In the present study none of the isolates obtained from media used for *Alkaligenes*, *Bacillus* and *Klebsiella* showed nitrogenase activity. N_2-fixing capacity of bacteria are sometimes lost during purification and storage, this could be the possible reason for low incidence of nitrogenase activity. The variation in ARA among the different isolates of *Azotobacter Derxia* and *Pseudomonas*-like organisms are due to strain differentiation, however, their range of activities are comparable with the results obtained by Gibson *et al.*, 1988. They observed nitrogenase activity of 6.6, 1.9 and 0.9 µg moles C_2H_4/bottle/24hrs for *Derxia gummosa*, *Pseudomonas* sp. 4B and *Azotobacter beijerinekia* veg.B, respectively. Three strains of *Azospirillum* sp. 5A, KG82 and DN64 showed ARA of 4.3, 6.9 and 11.0 µmoles C_2H_4/bottle/24hrs, respectively which appear to be very high as compared to the isolates of the present study (0.52 to 3.19 n moles/vial/hr).

4.1.2. Estimation of N_2-fixing capacity of isolates by kjeldahl method

When N_2-fixing capacity was measured at the cost of g^{-1} carbon source the results (Table-2) show that putative *Azotobacter*, *Derxia*, *Azospirillum* , *Pseudomonas* can fix 3.29 to 6.92, 3.29 to 11.51, 2.63 to 3.95 and 2.32 to 11.12 mg N g^{-1} of carbon source. The average of the data indicates that *Derxia* like organisms showed maximum activity (Average, 8.16mgN) follow by *Pseudomonas* (6.87mgN), *Azotobacter* (4.31mgN) and *Azospirillum* (3.29mgN) like organisms.

Table 2 : Acetylene reduction activity (ARA) & atmospheric N fixed / g of Carbohydrate source & growth promoting substance (IAA) production by various diazotrophs

Isolates	ARA (n mole. vial $^{-1}$ h^{-1})	mg N fixed g $^{-1}$ of C-source	IAA (µg ml^{-1})	Isolates	ARA (n mole. vial $^{-1}$ h^{-1})	mg N fixed g $^{-1}$ of C-source	IAA (µg ml^{-1})
A2	9.51	4.28	ND	D4	95.83	8.03	-
A3	28.53	4.94	ND	D5	208.83	9.54	-
A6	59.90	6.59	3.92	D6	104.62	8.43	-
A7	35.18	6.92	9.64	D7	140.62	3.29	2.23
A8	23.76	3.48	-	D8	15.62	ND	ND
A9	42.78	4.13	-	D10	91.14	6.58	4.67
A10	20.91	3.29	-	D12	114.58	11.51	-
A11	38.41	3.84	9.30	D13	135.41	8.85	-
A12	6.77	0.99	-	D16	85.93	ND	ND
A13	48.49	4.63	1.01	D18	203.12	8.87	-
A14	17.11	ND	ND	Ap3	1.56	3.29	8.44
A15	53.24	4.28	-	Ap5	0.52	2.63	-
A24	20.91	ND	ND	Ap18	3.19	3.95	-
A26	38.41	ND	ND	P3	412.49	11.12	-
D1	156.25	7.92	-	P4	298.42	7.17	-
D2	216.14	10.08	-	P12	54.62	2.32	12.50
D3	122.39	6.69	-				

Average of three replications. ND - Not determined. '-' , Negative.

Gupta, *et al.* (1992) showed that *Azotobacter* can fix atmospheric N @ 1.47 to 1.50 (Average, 1.49) mg N g^{-1} of carbon source, whereas, Gondotra *et al.* (1998) found the range as 13.3 to 21.6 mg N g^{-1} glucose. In the present study the activity is 3.29 to 6.92 mg N g^{-1} glucose. Thus the wide variation in N_2-fixing capacity of different isolates could be attributed to strain variation (Gupta and Tripathi, 1986). Nayak (1977) gave different explanation to the variation of N_2-fixing capacity of *Azospirillum* isolates. He found the range, 2.38 to 5.96 mg N g^{-1} carbon by the isolates obtained from soil amended with 20 kg N ha^{-1}, whereas, in case of 40 kg ha^{-1} the range was slightly higher i.e. 3.17 to 5.00 mg N g^{-1} of carbon source.

4.1.3. Screening based on IAA producing ability

Indole acetic acid producing ability was tested only for those isolates having acetylene reduction activities. Among thirty three diazotrophic isolates only eight showed IAA producing ability the quantity of IAA produced vary from 1.01 to 12.50 μgml^{-1} ; the maximum was recorded with P12 followed by A7, A11, Ap3 at the extent of 12.50, 9.64, 9.30, 8.44 μgml^{-1}, respectively .Previous workers also found indole acetic acid producing ability of *Azotobacter* (Gonzalez-Lopez *et al.*, 1986; Marting-Toledo *et al.*, 1988), *Azospirillum* (Zimmer *et al.*, 1991; Zimmer *et al.*, 1988 and Hartman *et al*, 1983) and *Pseudomonas* (Brown,1972). Results of the present study are comperable with the results obtained by Malik *et al.*,(1993) except *Pseudomonas*-like organism of our study. Out of 19 strains tested, he found 10 IAA producing strains and maximum was observed with *Pseudomonas* (35.7 $\mu g\ ml^{-1}$).

After critical evaluation of the test isolates with respect to their ARA and N_2-fixing ability in culture, none of the isolates appeared to be so

encouraging for their use under field condition. Moreover, in most of the standard schedules, recommendation of those culture of heterotropic bacteria (particularly *Azotobacter*) are generally made which have the ability of fixing at least 10mgN g^{-1} of sugar utilized (ISI,1970). Even then, the test cultures were not discarded only due to the facts that crop inoculation with diazotrophs does not necessarily mean only the amount of nitrogen fixed; it has certain other beneficial aspects like production of PGRs, biopesticidal effects and competition with undesirable rhizospheric microflora. In facts, the expression of such inoculation response is a cumulative effect of all probable benefits. Moreover, these isolates, being native of this particular region, would be the best adaptive ones for establishment and colonization.

4.2. Characterization of isolated diazotrophic bacterial strain

Out of the 98 isolates studied initially, eight diazotrophs were selected representing each of the predominant groups and these were characterized mostly under mesophilic and aerobic condition.

4.2.1. Morphological and cultural characteristics

Microscopic observations showed wide variations in shape like rod (P4 and P12), curved rod (Ap3 and Ap18), rod with rounded end (A6 and A7), rod to ovoid (D2) and ovoid (P3). Size of the isolates also vary from 1.6-2.4 x 0.4-0.8 µm to 3.1-4.6 x 2.0-2.4 µm and ovoid isolate has diameter of 1.6 to 2.7 µm. (Table-3). All isolates are gram -ve and nonspore former . Capsule was observed only in A6 and A7. Hanging drop suspensions showed that all diazotrophs are motile except isolate A6.

On Jensen's medium A6 produced irregular, smooth, slimy, opaque colonies and A7 gave round to irregular, smooth, low convex, gummy,

Table 3 : Morphological and cultural characteristics of different diazotrophic bacterial strains.

Characters	Diazotrophic Strains							
	A_6	A_7	Ap_3	Ap_{18}	D_2	P_3	P_4	P_{12}
Shape :	Rod with rounded end	Rod with rounded end	Curved rod	Curved rod	Rod to ovoid	Ovoid	Rod	Short Rod
Size	3.1-4.6x 2.0-2.4	3.2-4.2 x 1.7-2.3	2.3-3x0.9-1-1	2.1-2.7x 0.8-1.0	2.0-3.2x1.7-2.0	dia.1.6 to 2.7	1.8-3.2x0.6-1.1	1.6-2.4x0.4-0.8
Gram staining	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Endospore	-	-	-	-	-	-	-	-
Capsule	+	+	-	-	-	-	-	-
Motility	-	+	+	+	+	+	+	+
Colony morphology	Irregular, smooth, slimy, opaque colonies on J.M.	Round to irregular, smooth, low convex, gummy, glistening, opaque colonies on J.M.	Irregular, wrinkled, umbonate light pink colonies on BMS agar and on Na-malate medium it produces small, white, dense colony.	Irregular, wrinkled, umbonate light pink colonies on BMS agar and on Na-malate medium it produces small, white, dense colony.	Round, entire margin, smooth, raised, creamy white colony.	Round, entire margin, smooth, slightly raised gummy, pale yellow colony.	Round, entire margin, smooth, covex, gummy, transparent colony.	Round, entire edge, slightly convex, white, glistening and become dirty white in old culture.
Pigment production	Pale yellow to light brown nondiffusible pigment on Fe-free glucose medium and diffusible brown pigment in presence of benzoate.	Dark brown to black nondiffusible pigment on Fe-free glucose medium and light brown pigment in presence of benzoate.	Pink pigment on BMS agar.	Pink pigment on BMS agar.	Not produced	Not produced	Bright yellow pigment produced in old culture.	Not produced

Symbol : '+', Positive; '-', Negative.

glistening, opaque colonies. Colony morphology of Ap3 and Ap18 showed irregular, wrinkled, umbonate light pink colonies on BMS agar and small, white, dense colonies on Na-malate medium, Both D2 and P3 produced round, entire margin, smooth, raised colonies with the difference that D2 produced creamy white colonies and P3 produced pale yellow colonies on their isolation media. Colony of P4 was round, entire margin, smooth, convex, gummy and transparent, whereas, P12 was round, entire edge, slightly convex, white, glistening and became dirty white in old culture.

Studies on pigment production showed that on Fe-free glucose medium A6 produced pale yellow to light brown nondiffusible pigment, whereas, A7 produced dark brown to black pigment. In presence of benzoate, A6 also gave diffusible brown pigment. Isolates Ap3 and Ap18 produced pink pigment on BMS agar. Isolates D2, P3 and P12 did not show any pigment production, whereas, P4 produced bright yellow pigment in old culture.

Observations on shape, size, capsule, colony morphology and pigment production on JM, and tallying with *Bergey's Manual of Systematic Bacteriology* (Tchan and New, 1984) depicted that isolates A6 and A7 could be *Azotobacter beijerinckia* and *A. chroococcum,* respectively. However, nonmotile character is present in both *A. bejirinckia* and *A. nigricans,* but no strain of *A. nigricans* can produce brown diffusible pigment like *A. beijerinckia.* Colony morphology and pigment production of isolates Ap3 and Ap18 resemble the *Azospirillum* as described by Dobereiner *et al.*, 1976 and Dobereiner and Baldani, 1979. Other morphological characters like size, shape gram staining and motility also confirm their genus character. Colony morphology of isolate P12 are similar to N_2-fixing *Pseudomonas* described by Barraquio and Watanabe, 1981. Shape, size, motility and other morphological characters

also resemble *Pseudomonas* (Watanabe *et al.*, 1987 and Barraquio *et al.*,1983). This isolate did not produced pigment; Barraquio *et al.*, (1983) also reported that strains H8 and KLH76 represent the pigment and nonpigment producing isolates, respectively.

4.2.2 **Physiological characteristics**

Selected biochemical tests were carried out to define the organism and variation was observed among the eight isolates. Oxides test showed positive result with all the isolates except P4 and catalase was also produced by most of the diazotrophs. Negative Methyl-Red and Voges- Proskauer result were observed in all the organisms. Hydrolysis of starch casein, gelatin and urea were done. Starch was hydrolyzed by A7, D2 and P3; none of the isolates hydrolyzed casein; gelatin was hydrolyzed only by P12, whereas, isolates Ap3, Ap18 and D2 showed urease activity. Conversion of NO_3 to No_2 was observed with all the isolates except D2 and isolates A6, A7, Ap3, P3 and P12 produced indole. Utilization of citrate was positive with A6, A7, Ap3, A18 and P12 , weak positive with P4 and negative with D2 and P3 (Table-4).

Physiological characters A6 and A7 resembles those of genus *Azotobacter*, only strain differentiation was observed with amylase activity. Physiological characters of Ap3 and Ap18 are almost similar and both are similar to *Azospirillum* according to *Bergey's Manual of Systematic Bacteriology* (Krieg and Dobereiner,1984). Considering the oxidase, Catalase, Methyl Red, Voges Proskauer (Barraquis *et al.*, 1983), gelatin and starch hydrolysis (Palleroni, 1984) P12 resemble the genus *Pseudomonas*. However, Barraquio *et al.*, (1983) observed negative indole test for strain H8 and KLH76 .

4.2.3 . **Utilization of organic acid , simple sugar and sugar alcohol**

Utilization of seven organic acids, seven simple sugars and three sugar alcohols were tested by all the eight isolates (Table-5). Isolates A6 utilized all

Table 4 : Physiological characteristics of various nitrogen fixing organisms

Characters	Diazotrophic Strains							
	A6	A7	Ap3	Ap18	D2	P3	P4	P12
Oxidase	+	+	+	+	+	+	-	+
Catalase	+	+	+	+	W	+	+	+
MR	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-
Hydrolysis of *Starch*	-	+	-	-	+	+	W	-
Casein	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	+
Urea	ND	ND	+	+	+	-	-	+
NO_3^- to NO_2^-	+	+	+	+	-	+	W	+
Indole	+	+	W	-	-	+	-	+
Utilization of Citrate	+	+	+	+	-	-	W	+

Symbols : '+' , positive ; '-' , negative ; 'W' , weak positive ; 'ND' , not determined ; 'MR', Methyl Red ; 'VP', Voges-Proskauer.

Table 5 : Utilization of organic acid , simple sugar and sugar alcohol as C-source by isolated N_2 - fixing organisms

C - source	Diazotrophic Strains							
	A6	A7	Ap3	Ap18	D2	P3	P4	P12
Organic Acids :								
Acetate	+	+	-	-	+	-	-	+
Malate	+	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	ND	+	+
Benzoate	+	+	-	-	-	-	-	-
Fumerate	+	+	+	+	+	+	-	+
Propionate	+	+	+	+	-	-	-	W
Lactate	+	+	+	+	-	W	+	+
Simple Sugars :								
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	W	-	W	-	+	+
Fructose	+	+	+	+	W	+	ND	+
Arabinose	+	+	W	-	+	+	-	-
Sucrose	+	+	-	-	+	+	+	+
Maltose	+	+	-	-	+	+	+	W
Lactose	+	+	-	-	+	+	W	W
Sugar alcohol :								
m - Inositol	-	-	-	-	+	+	W	W
Mannitol	-	+	-	+	+	+	+	+
Glycerol	-	+	+	+	+	+	W	+

Symbols : '+' , positive ; '-' , negative ; 'W' , weak positive ; 'ND' , not determined.

organic acids and simple Sugars except three sugar alcohol, whereas, except *m*-inositol all carbon sources were catabolized by A7. Similarity in utilization pattern was noticed in case of Ap3 and Ap18 except mannitol. Both can utilize malate, succinate, propionate, lactate, glucose, fructose and glycerol. Among the 17 carbon compounds D2 could not utilize benzoate, propionate and lactate, whereas, P3 did not show any visible growth on acetate, propionate, benzoate and galactose. P4 was positive for eight, weak positive for three and negative for five C-sources. Except benzoate and arabinose P12 could metabolized all the tested compounds of which maltose, lactose and *m*-inositol were weakly utilized.

Isolates A6 and A7 that resemble *A. beijierinckia* and *A. chroococcum*, respectively, depicted similar type of carbon utilization pattern and it is important to mention that propionate and benzoate were utilized by A6 which are not metabolized by *A. nigricans*. Utilization pattern of organic carbon compound indicates the strain differentiation between Ap3 and Ap18. It was also observed that both can utilize glucose and mannitol, which depicted that these strains could be *A. lipoferum* as because *A. brasilense* can not utilize these two compound as sole carbon source.

During characterization of isolates, a preliminary attempt was taken to identify eight selected diazotrophs. Isolate A6, A7, and P12 were identified as *Azotobacter beijerinckia, A. chroococcum* and *Pseudomonas*, respectively, whereas both Ap3 and Ap18 were *Azospirillum lipoferum*. However, three isolates remain unidentified which need some basic studies to identify them. Considering our main objectives to study the effect of diazotrophs in N-economy of lowland rice soils, we did not proceed further to identify these organisms.

4.3. Screening of effective ' diazotroph x cultivar' combination

Pot experiment was conducted with the objective to screen varieties, diazotrophs and variety diazotroph combinations that support better BNF. Three rice genotypes were inoculated with eight diazotrophic strains isolated initially and observations were recorded for number of tillers, dry shoot weight N-uptake and microbial population.

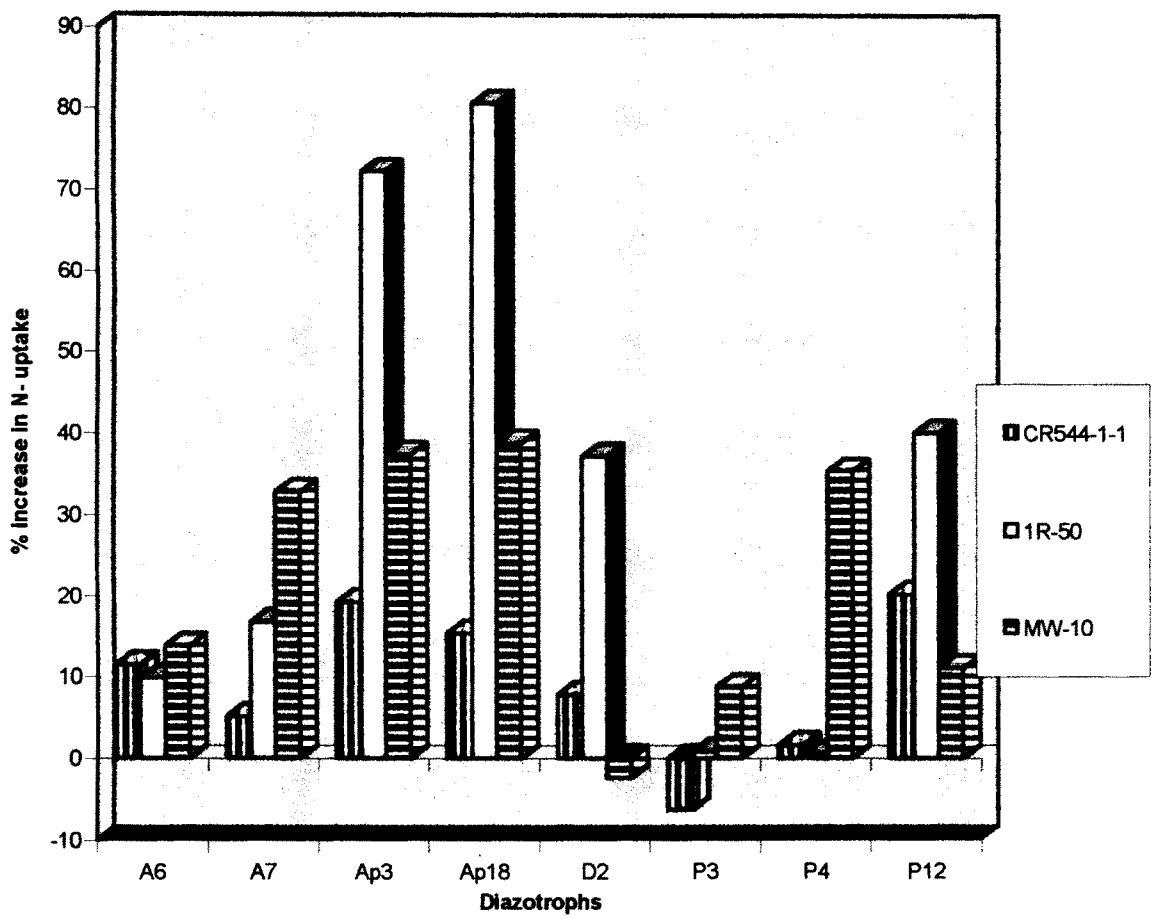
Number of tillers/pot vary significantly with rice varieties and diazotrophs. Effect of diazotrophs show that number of tillers vary with least significant difference over control only for A6, A7, Ap3, Ap18 and P3 (Table-6). The maximum effect was observed for isolate A7. Diazotrophic effects, expressed in percentage over control, ranged from 1.76 to 10.35, 12.78 to 30.33 and -6.38 to +21.32 for CR-544-1-1, IR-50 and MW-10, respectively, shows the superiority of, R-50 over other two cultivars. Maximum diazotroph genotypes interaction was observed in variety, CR-544-1-1 and IR-50 with A7 and in MW-10 with A6.

Dry weight of shoot also vary significantly with varieties, diazotrophs and their interactions. Among the diazotroph A7 produced maximum benefit followed by Ap18, A6 and D2 (Table-7). Among the varieties, effect of diazotrophs was more pronounced in IR-50. Percentage increase of shoot dry weight over control indicates that it varies from 25.97 to 55.00 in IR-50, -9.71 to +43.42 in MW-10 and -14.75 to 12.62 in CR-544-1-1. Interactions also depict that CR-544-1-1 showed maximum effect when inoculated with Ap18 followed by A6 and P12; in case of IR-50 it was with Ap18 followed by A7 and Ap3. MW-10 showed better effect with A6 and A7.

Significant variation in plant N-uptake were observed for varieties, N_2-fixers and interactions. Considering the variety averages, the ranking of N-accumulation induced by eight diazotrophs was in the decreasing order of

Ap18, Ap3, P12, A7, A6 (Table-8). Observations on percentage increase over control revealed that the effect was maximum with IR -50 (0.57 to 80.6) followed by MW-10 (-2.33 to +38.76) and CR-544-1-1 (-6.29 to +20.20)(FIG 1)ions between varieties and diazotrophs depicted that the best combination in respect of N-uptake was 'IR-50 x Ap18' followed by 'IR-50 x Ap3' and ' IR-50 x P12'.

FIG1: Percent increase in N-uptake by three rice varieties inoculated with diazotrphs over uninoculated control



Rhizosphere and rhizoplane samples of uninoculated plant samples were analyzed to assess varietal effect on aerobic and microaerophilic diazotrophs. It was observed that for both the groups IR-50 harboured maximum population in rhizosphere and rhizoplane(Table-9).

Table :6 Performance of three rice varieties inoculated with various diazotrophic bacterial strains

Treatment	Number of tillers/ pot			
	CR-544-1-1	IR-50	MW-10	Mean
Control	9.66	12.66	7.83	10.06
A_6	10.16 (5.17)	16.16 (27.64)	9.50 (21.32)	11.94
A_7	10.66 (10.35)	16.50 (30.33)	8.83 (12.77)	12.00
Ap_3	10.00 (3.52)	15.50 (22.43)	8.16 (4.21)	11.22
Ap_{18}	10.50 (8.69)	16.00 (26.38)	9.33 (19.15)	11.94
D_2	10.50 (8.69)	14.66 (12.78)	7.33 (-6.38)	10.50
P_3	10.33 (6.93)	15.66 (23.70)	8.33 (6.38)	11.44
P_4	10.00 (3.52)	14.33 (13.19)	7.66 (-2.17)	10.66
P_{12}	9.83 (1.76)	14.66 (12.78)	8.33 (12.77)	10.94
Mean	10.07	15.13	8.37	
	Variety	Diazotroph	Variety x Diazotroph	
S.Em±	0.20	0.34	0.59	
$CD_{0.05}$	0.55	0.95	NS	

NS - Not significant. ; Figure within parentheses indicates % increase over control.

Table 7 : Response of rice varieties inoculated with different diazotrophs on their dry matter production

Treatment	Shoot dry weight /pot (g)			
	CR-544-1-1	IR-50	MW-10	Mean
Control	8.95	8.20	7.00	8.05
A6	9.88 (10.39)	10.33 (25.97)	9.41 (34.42)	9.87
A7	9.41 (5.13)	12.68 (54.63)	8.66 (23.71)	10.25
Ap3	9.68 (8.15)	12.53 (52.80)	7.70 (10.00)	9.97
Ap18	10.08 (12.62)	12.71 (55.00)	7.48 (6.85)	10.09
D2	8.96 (0.11)	11.78 (43.66)	7.23 (3.28)	9.32
P3	7.63 (-14.75)	11.73 (43.04)	6.32 (-9.71)	8.56
P4	9.35 (4.47)	11.20 (36.58)	7.80 (11.42)	9.45
P12	9.71 (8.49)	10.93 (33.29)	8.00 (14.28)	9.55
Mean	9.30	11.34	7.73	-
	Variety	Diazotroph	Variety x Diazotroph	
S.Em±	0.19	0.34	0.59	
$CD_{0.05}$	0.55	0.95	1.66	

Figure within parentheses indicates % increase over control

Table 8: N-uptake of different rice varieties inoculated with diazotrophs

Treatment	N-uptake (mg N / pot)			
	CR-544-1-1	IR-50	MW-10	Mean
Control	69.15	88.15	60.70	72.66
A6	76.75 (11.0)	96.86 (9.88)	69.06 (13.77)	80.89
A7	72.70 (5.13)	102.88 (16.71)	80.60 (32.78)	85.39
Ap3	82.43 (19.20)	151.78 (72.18)	83.38 (37.36)	105.86
Ap18	79.78 (15.37)	159.20 (80.60)	84.23 (38.76)	107.73
D2	74.55 (7.80)	120.93 (37.18)	59.28 (-2.33)	84.92
P3	64.80 (-6.29)	88.86 (0.81)	66.05 (8.81)	73.23
P4	70.28 (1.63)	88.65 (0.57)	82.51 (35.93)	80.48
P12	83.00 (20.20)	123.45 (40.04)	67.41 (11.05)	91.28
Mean	74.83	113.44	72.58	
	Variety	Diazotroph	Variety x Diazotroph	
S.Em±	1.35	2.34	4.06	
$CD_{0.05}$	3.79	6.56	11.37	

Figure within parentheses indicates % increase over control.

Table 9 : Aerobic and microaerophillic diazotrophs in rhizosphere and on rhizoplane of three rice cultivars

Variety	Putative diazotrophs on CCM (x 10^5)	*Azotobactor* (x 10^2)	MPN of *Azospirillum* like organism (x 10^5)	MPN of putative *Pseudomonas* (x 10^6)
CR-544-1-1a	32.7	12.4	7.2	17.8
b	28.0	-	11.5	22.8
IR-50 a	65.8	28.7	18.6	67.12
b	132.6	-	21.6	92.6
MW-10 a	30.5	5.6	7.9	15.1
b	35.2	-	11.5	17.8

a - CFU per gram of dry rhizosphere sample.

b - CFU per gram of dry rhizoplane sample .

Azotobacter was found only in rhizosphere sample, none of the rhizoplane sample gave *Azotobacter* colony. MPN count of *Pseudomonas*-like organisms was more as compared to *Azospirillum.* Van Holm *et al.,* (1994) reported that the number of dinitrogen fixing bacteria in the rhizosphere of three rice cultivar was not different. However, cultivar differences in associative BNF were reported by Roger and Ladha (1992). In this study, differences were observed, both in rhizosphere and rhizoplane. In the present study it was also observed that population of *Azospirillum* and *Pseudomonas*-like organisms were ten fold lower than the observation recorded by Van

Holm *et al.,* (1994) in Srilankan rice field. Rice cultivars having higher rot biomass (like IR-50) exerted more rhizospheric effect. Similarly, poor organic matter status and poor soil nutrient status of Terai soil is responsible for lower microbial population .

Considering the earlier reports about the correlation of total N-uptake and plant biomass with associative N_2-fixation among different genotypes (Ladha *et al.*, 1999) it may be suggested to pay more emphasis on N-uptake and plant biomass to select effective diazotroph and rice cultivar. Similar findings were also reported on the basis of N-balance, ARA and N dilution estimates (App *et al.*, 1986; Ladha *et al.*, 1987; Shrestha and Ladha,1996). So, in the concluding part of this experiment, it can be highlighted that IR-50 was the best variety in respect of plant biomass, N-uptake and stimulation of diazotrophic population in the rhizosphere. Irrespective of varieties studied, *Azospirillum* like organism has emerged out as the best inoculum followed by P12 and A7. Among the 'Variety x Diazotroph' combinations, *Azospirillum, Pseudomonas* and *Azotobacter* appeared to be the best when inoculated to IR-50. In the present study, the isolates D2, P3 and P4 have failed to produce any encouraging result although the same have showed better ARA activities under cultural condition. On the contrary, p18 showed better result in spite of having low ARA. Mechanisms contributing such differences included (1) Specific nutrient uptake rates (Per unit root surface), (ii) modification of the rhizosphere by root metabolites, and (iii) exudations, including the stimulation of associative N_2-fixation in the rhizosphere (Ladha *et al.*, 1998). The better result of IR-50 can be explained with its higher root biomass as compared to CR-544-1-1 and MW-10, which is the main cause for derepression of nitrogenase enzyme through depletion of available nitrogen by a vigorous root system.

Thomas-Bauzon *et al.* (1982) introduced a new approach to select rice 'cultivar x diazotroph' combination for better N_2-fixation using axenic rice seedling in Pankurst tube inoculated with diazotrophs without providing 'C' and 'N' from outside. This approach does not hold good always (Charyulu *et al* .,1985) probably due to edaphic factor which was not considered during selecting efficient combination of 'cultivar x diazotroph'. Here, it seems logical to report the selection of 'cultivar x diazotroph', combination using unsterilized soil, thereby to consider environmental factors along with the varieties as well as diazotrophs.

4.4.Performance of diazotrophs in different agroecological zones

Eight diazotrophs (Table-10 , 11 & FIG 2) were tested in Terai zone under net house condition, and in New Alluvial , Red Laterite , and Coastal Saline zone under field condition. A second set of ten N_2-fixers were also evaluated in New Alluvial zone under field condition. It was observed from pot experiment with Terai soil that the result is significant only for effective tillers/pot, Panicle length, grain weight and straw weight. Significant difference was not observed among the diazotrophs in case of parameters like panicle length and grain weight. But for both the parameters Ap18 appeared to be the best. However, for straw weight, D2 recorded maximum response. All the experiments conducted under field condition in different zones showed significant variation among the treatments .In New Alluvial zone for both grain and straw yield, response was maximum with D2 followed by A6 and A7. Isolate P4 showed better performance in respect of grain yield in Red Laterite zone and for straw yield also P4 was the best inoculum followed by A6 and A7. In Coastal Saline zone, for grain yield P4 appeared to be the best followed by P12 and D2, whereas, for straw yield D2, P12 and P4 showed

Table 10 : Response of rice variety IR-50 to carrier based inoculum of various diazotrophic bacteria in Terai soil under nethouse condition (Kharif - 1997)

Diazotroph	Plant height (cm)	Effective tillers /pot	Panicle length (in cm)	Number of grain/panicle	1000 grain weight (g)	Grain weight per pot (g)	Straw weight per pot (g)
Control	59.66	11.83	17.83	93.83	18.68	15.05	8.30
A_6	62.83	13.16	19.50	99.50	19.60	19.00	11.80
A_7	63.00	13.33	19.33	99.66	19.90	19.48	11.15
Ap_3	61.66	12.83	18.50	94.83	19.33	19.56	10.16
Ap_{18}	62.33	13.83	19.66	99.50	19.25	19.96	11.35
D_2	63.33	13.33	19.83	103.33	19.28	17.50	12.13
P_3	60.50	14.50	19.33	94.50	19.85	18.11	11.50
P_4	60.66	15.66	19.16	103.83	19.23	17.48	11.26
P_{12}	60.83	15.50	19.08	101.00	19.41	19.05	10.45
S. Em±	1.18	0.70	0.45	3.34	0.51	0.90	0.52
$CD_{0.05}$	NS	1.99	1.29	NS	NS	2.59	1.48

Table 11 : Performance of different diazotrophic bacterial strains as inoculant in the lowland rice field of three agro-ecological zones of West Bengal (Kharif,1997)

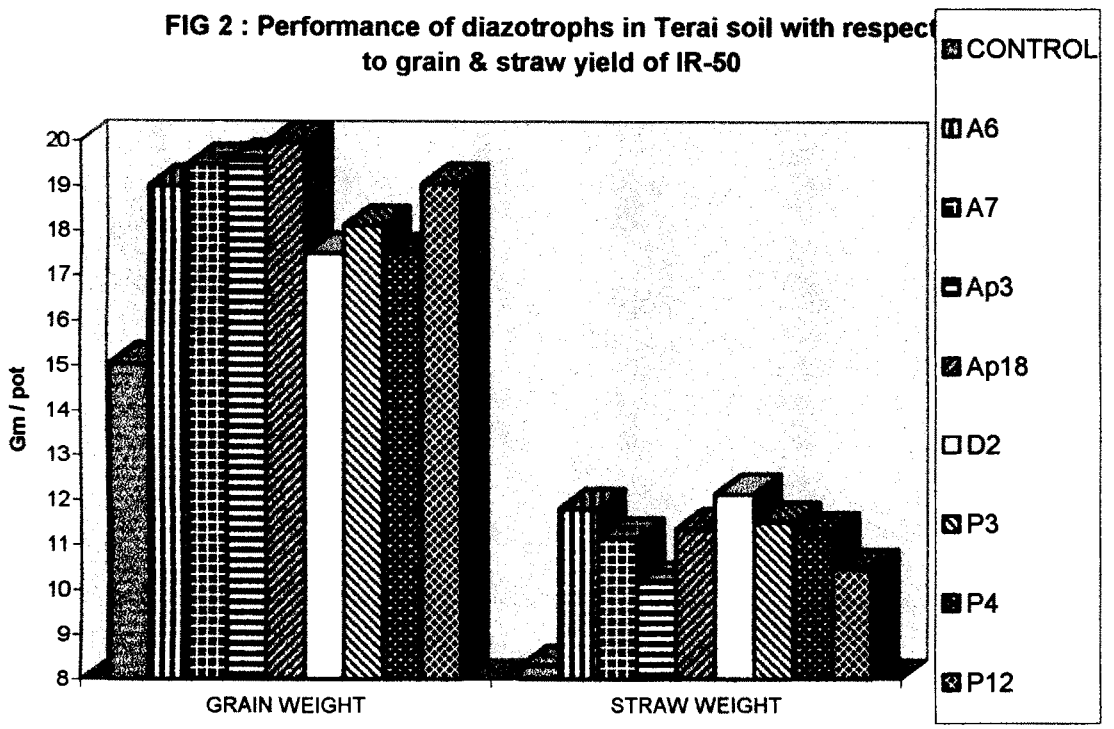
Diazotrophic strain	Grain yield (t ha $^{-1}$)			Straw yield (t ha $^{-1}$)		
	New Alluvial Zone	Red Laterite Zone	Coastal Saline Zone	New Alluvial Zone	Red Laterite Zone	Coastal Saline Zone
Control	3.39	2.40	2.87	3.74	2.82	4.06
A6	4.00	3.30	3.75	4.13	3.63	5.25
A7	3.89	3.06	3.56	4.04	3.55	6.12
AP3	3.83	2.65	3.00	3.77	3.13	5.51
AP18	3.45	2.71	3.38	3.67	3.05	5.86
D2	4.06	2.93	3.88	4.50	3.33	6.60
P3	3.35	3.15	3.81	3.69	3.45	5.46
P4	3.79	3.30	4.56	3.89	4.00	6.16
P12	3.58	2.81	3.93	3.75	3.23	6.51
S. Em ±	0.07	0.08	0.10	0.09	0.13	0.22
CD $_{0.05}$	0.22	0.23	0.30	0.27	0.38	0.64

Table 12 : Screening of diazotrophs on the basis of their effect on growth and yield attributes of lowland rice in New Alluvial zone(kharif, 1996)

Diazotrophs	Plant height* (cm)	Root length* (cm)	Root dry weight* (g)	Shoot dry* weight/hill (g)	Number* of tiller/hill	Number of effective tiller/hill	Panicle length (cm)	Number of grain / Panicle	Grain yield (t ha^{-1})
A7	75.6	14.6	3.3	11.7	18.0	14.6	20.8	182.0	5.4
A9	79.3	17.0	3.4	13.3	17.6	11.3	21.5	173.0	6.7
A11	79.3	15.3	3.1	13.9	19.6	12.3	22.6	205.6	6.3
A13	82.3	21.0	4.5	16.4	18.6	10.6	20.5	177.6	4.6
A15	80.3	17.6	3.8	12.4	17.0	10.0	20.8	129.6	5.3
D2	81.6	15.6	3.8	13.5	14.0	9.3	19.0	125.0	5.2
D5	86.0	19.0	3.8	16.7	16.0	10.3	20.1	147.6	5.4
D18	80.6	15.6	3.2	12.1	15.3	11.3	20.5	172.0	5.5
MP251	83.6	16.0	3.2	14.1	14.6	13.6	21.5	168.6	6.5
BU351	84.6	17.0	6.4	18.6	18.6	13.0	19.5	148.6	5.8
S.Em±	2.49	0.72	0.51	1.11	1.21	0.78	0.66	9.04	0.31
CD 0.05	NS	2.96	NS	4.56	NS	NS	NS	36.81	1.25

*Observations recorded 45 DAT; NS-not significant.

the same effect. A second set of isolate (Table 12) were compared in New Alluvial zone. No appreciable difference was observed among the isolates in inducing variation in respect of plant height- the parameter being an intrinsic property of the plant. The isolates also failed to bring about any



variation in respect of root dry weight, number of tillers and panicle length. Maximum number of effective tillers was induced significantly with A7 followed by two *Azospirillum* isolates MP251 and BU351. Root length and dry weight of shoot varied considerably due to inoculation with different isolates.

Maximum root length and maximum dry weight of shoot were stimulated by A13 and B351, respectively. The isolates also bring about significant variation in respect of number of grains per panicle and total grain weight . The isolate, A11 appeared to be the best in respect of first parameter but the total grain weight was maximum with A9, the isolate which

incidentally did not score very high in respect of any of the yield contributing parameters studied. Apparently, there did not exist any correlation between the final yield and any of the other plant parameters considered in this study. It seems therefore, that the final yield is the resultant of cumulative effects of different parameters.

Performance of eight selected diazotrophs varied considerably in different agricultural zones of West Bengal. In Terai zone pot experiment showed that grain yield scored better when inoculated with *Azospirillum* (AP18). In Red Laterite and Coastal Saline zone, maximum response in respect of grain and straw yield was exhibited by an isolate of *Pseudomonas* like organism (P4). Two sets of isolate, evaluated in New Alluvial zone, depicted that grain yield of rice was maximum with *Derxia* (D2) and *Azotobacter* (A9) like organisms.

Thus it becomes evident from the present study that while selecting diazotroph strains as ideal rice inoculants, emphasis should be given on using region specific best adapted strains no matter as to whether the strains perform well or not in the laboratory. In deed, N_2-fixing ability of a diazotroph strain is its intrinsic property but its proliferation and activity are much dependent on different environmental factors of a particular ecological situation to which the strain is exposed. Hence, none of the "effective strains" of diazotroph can be recommended for universal application unless it is tested against a particular crop under a particular agro-ecological situation.

4.5. Effect of inoculation methods on effectiveness of biofertilizers

Seed bed inoculation, seed inoculation and seedling inoculation were evaluated and these were also compared with uninoculated control under field condition. Different methods of inoculation failed to induce variation in

respect of plant height, dry weight of shoot, number of effective tillers, panicle length and number of grains per panicle. However, there had been significant variation among the treatments in respect of root length and dry weight of root (Table-13). Seedling inoculation appeared to be the best in this regard, although increase in root dry weight due to seedling inoculation was not significant in comparison to control. Number of tillers per plant and total grain weight experienced significant variation due to different inoculation methods. Here again, seedling inoculation stood to be the best of all. Hence, it is suggested that effective inoculation of rice can only be achieved through seedlings inoculation method. The present findings are also in agreement with the previous reports made by Gopalaswamy and Vidhyasekaran(1987a, b). They also observed that in a comparison of seven combinations of seeds, seedlings and soil inoculation , a significant increases in yield was obtained only when seedlings and soil were inoculated.

In case of rice, seed inoculation may not be as effective as it is seen in case of legumes. Colonization of the heterotrophs around the rice root zone through seed inoculation is not a simple phenomenon and may require adequate time for establishment. But in case of seedlings inoculation, the bacteria got adsorbed all through the rhizoplane areas which render greater chance of bacterial colonization throughout the root.

4.6. Performance of mixed inocula over single inoculum on rice var. IR-50 in Terai soil

An attempt was made to compare the effectiveness of mixed inocula over single inoculum. Out of the five isolates studied two were isolated on aerobic condition and rest three under microaerophilic condition. Isolate Ap18 was included in all the mixed inoculation treatments considering its

Table 13 : Effect of inoculation methods on various growth and yield parameters of lowland rice (kharif, 1996)

Treatment	Plant height* (cm)	Root length* (cm)	Root dry weight* (g)	Shoot dry* weight/hill (g)	Number* of tiller/per hill	Number of effective tiller/hill	Panicle length (cm)	Number of grain / Panicle	Grain yield (t ha^{-1})
Control	85.0	18.1	5.8	14.9	18.0	10.0	21.3	177.0	6.9
Seed bed inoculation	86.3	18.8	2.8	14.3	19.0	11.0	22.5	185.0	7.3
Seed inoculation	87.6	19.6	5.5	15.5	21.3	11.6	21.3	179.0	6.8
Seedling inoculation	92.3	21.3	8.2	16.3	23.0	11.6	21.0	194.0	8.3
S.Em ±	3.54	0.53	0.77	1.54	0.74	0.67	0.68	4.08	0.17
$CD_{0.05}$	NS	2.68	5.33	NS	2.57	NS	NS	NS	0.60

*** Observations recorded at 45 days after transplantation , 'NS' - not significant**

better performance in 'cultivar x diazotroph' screening experiment. Results of mixed inocula were not significant for parameters like plant height, panicle length and 1000 grams weight (Table-14). However, it was observed that all the mixed inoculations showed higher tiller number than single inoculation, maximum being with Ap18+A7 followed by Ap18+P12 and Ap18+P3. Among these three combinations, only Ap18+A7 differs with least significant difference from Ap18 and A7. Though the observation on number of grains /Panicle does not show the superiority of Ap18+A7, it could induce maximum straw and grain yield. In case of straw, Ap18+A7 gave significantly higher yield than any of the single and mixed inoculation followed by Ap18+P3 and Ap18+D2. For grain weight again this combination was followed by Ap18+P12 and Ap18 +D2 (FIG 3).

FIG 3 : Comparison between single & mixed inoculation with respect to grain weight of IR-50 in Terai soil

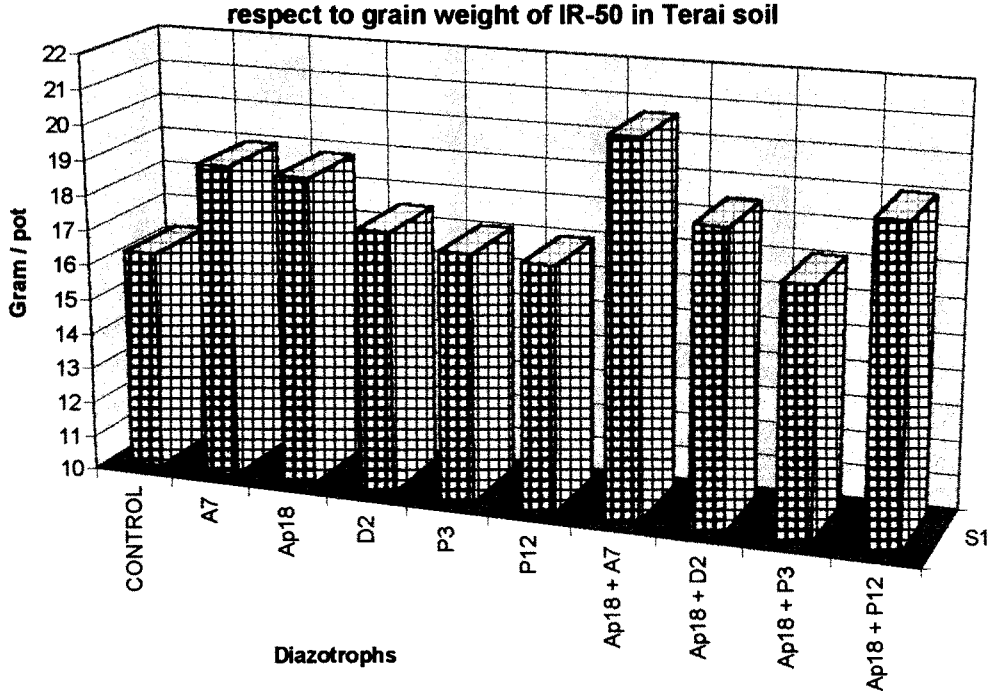


Table 14 : Performance of mixed inocula over single inoculum on rice var. IR-50 in Terai soil.

Treatments	Plant height (cm)	Effective tiller /pot	Panicle length (cm)	Number of grain / Panicle	1000 grain weight (g)	Grain weight /pot (g)	Straw weight /pot (g)
Control	59.67	13.50	18.58	89.83	18.98	16.25	10.40
A7	61.00	14.33	19.58	101.66	19.56	18.96	10.62
Ap18	61.83	13.83	18.75	101.50	19.63	18.83	11.10
D2	61.00	14.50	19.57	95.67	19.25	17.51	10.53
P3	60.00	14.16	19.00	94.67	20.93	17.11	10.00
P12	60.33	13.83	19.00	98.66	19.12	17.00	9.88
Ap18+A7	61.34	16.50	19.67	92.17	19.85	20.68	12.71
Ap18+D2	61.00	14.67	20.25	99.83	20.38	18.46	11.42
Ap18+P3	60.67	15.00	19.83	101.67	20.02	17.11	11.60
Ap18+P12	60.00	15.16	19.08	100.00	19.70	19.02	11.20
SEm±	1.22	0.64	0.45	3.65	0.57	1.13	0.51
$CD_{0.05}$	NS	1.81	NS	10.37	NS	3.23	1.44

Population of aerobic and microaerophilic N_2-fixers in rhizosphere, measured at maximum tillering stage also vary among the treatments. For aerobic organisms Ap18+D2, Ap18+P3 and Ap18+P12 showed maximum proliferation as compared to single inoculation (Table-15).

Table 15 : Aerobic and microaerophilic diazotrophs in rhizosphere of IR-50 inoculated with single & mixed inocula

Treatment	Putative aerobic* diazotrophs on CCM (x 10^5)	Putative microaerophilic* diazotrophs in glucose yeast extract medium(x 10^6)
Control	14.8	7.4
A7	38.3	17.3
Ap18	25.8	14.4
D2	33.4	16.9
P3	35.4	42.3
P12	29.3	37.9
Ap18 + A7	35.2	22.0
Ap18 + D2	41.2	40.7
Ap18 + P3	39.3	101.8
Ap18 + P12	38.4	103.8

* Population is expressed on dry weight basis

However, total microaerophilic diazotrophs proliferated only in case of rhizosphere treated with Ap18+P3 and Ap18+P12. These observations showed the poor survival of aerobic and microaerophlic diazotrophs in

Ap18+A7 treated rhizosphere and fail to justify its better effect as compared to other mixed inoculations.

It can be concluded from this experiment that a mixed inocula comprising microaerophilic and aerobic diazotrophs may have some synergistic effect in respect of efficiency as compared to single inoculation. This can be explained by the experiment of Khammas *et al.*, (1993). They observed significantly higher ethylene production (1930 n moles C_2H_4/plant/day) in spermosphere model. This kind of observations can be attributed to the metabolic association between the inoculated strain.

4.7. Role of organic matter on the effectiveness of biofertilizer (N_2 -fixer)

Experiments were conducted at Chakdah, New Alluvial zone and Kakdwip, Coastal Saline zone to assess the performance of biofertilizer in soil enriched with FYM. It was observed that at Chakdah, grain and straw yield vary significantly with graded N-levels having highest yield at 100 kg N/ha (Table-16). Effect of FYM also vary significantly and maximum yield of grain and straw were recorded when FYM applied twice in a year as compared to single and alternate year application. Interactions between nitrogen and various levels of organic matter enrichment depict significantly higher grain and straw yield when nitrogen interact with highest level of organic matter enrichment. This experiment was repeated in kharif season in New Alluvial zone with the similar treatments and showed significant effect of FYM, N and the interaction of both on grain and straw yield(table-17). In kharif season nitrogen at 60 and 80 kg/ha produced statistically same straw and grain yield, however, grain and straw yield at different FYM enrichment level vary significantly with least significant difference. Interactions also showed that

highest level of positive interaction was observed when N interact with FYM applied twice in a year. But here also yield obtained at 30 kg, 60 kg and 90 kg N/ha did not vary significantly. Experiment conducted in Coastal Saline zone showed that effect of nitrogen vary significantly for number of tillers, straw and grain yield, and for all the parameters effect of FYM was found insignificant (Table-18). However, FYM could increase number of tillers, straw and grain yield over control. Interactions showed that number of tiller was significantly higher when FYM interact with 100 kg N/ha, however, straw and grain yield did not vary significantly in interaction studies.

In a separate experiment at New Alluvial zone, different sources of organic matter was used along with biofertilizer. It was observed that maximum dry matter production was recorded when the soil was amended with rabbit litter, application of poultry litter induced maximum number of tillers, whereas, sheep litter application resulted maximum length of the panicle (Table-19). Improved grain weight was recorded by the application of all the organic sources used, although the differences appeared to be statistically insignificant. Maximum grain weight was obtained when the soil was amended with rabbit litter which might be attributed to its fast decomposing property in the soil.

Heterotrophic N_2-fixing bacteria fix N_2 biologically at the expense of energy derived from carbon sources under cultural condition. N_2-fixation by such heterotrophs under field condition depend much on available carbon in the soil by way of incorporation of straw, crop residues, organic manures etc. (Havelka *et al.*, 1982; Roper and Halsall, 1986). In view of above an attempt was made in present study to ascertain the extent of benefit of BNF by amending soil with organic matter even at higher doses of inorganic N. The results clearly indicate a definite increase in the crop yield when biofertilizer

Table 16 : Effect of organic matter (FYM) application on the effectiveness of N_2-fixing biofertilizer in managing nitrogen nutrition in boro rice (in New Alluvial zone)

Treatments	Straw yield (t ha^{-1})					Grain yield (t ha^{-1})				
	N_{70}	N_{80}	N_{90}	N_{100}	Mean	N_{70}	N_{80}	N_{90}	N_{100}	Mean
FYM-applied once/year	7.20	7.33	7.60	8.73	7.71	5.66	5.86	6.06	6.80	6.10
FYM-applied twice/year	7.73	8.53	8.66	8.73	8.41	6.93	7.86	8.26	8.73	7.95
FYM-applied every alternate year	6.86	7.06	7.20	8.20	7.33	5.60	5.73	5.76	6.60	5.92
FYM-not applied	6.40	6.80	7.46	8.40	7.27	5.53	5.53	6.20	6.53	5.95
Mean	7.05	7.43	7.73	8.51		5.93	6.25	6.57	7.16	
	FYN	N	FYM x N			FYN	N	FYM x N		
S.Em±	0.07	0.08	0.17			0.06	0.05	0.11		
CD $_{0.05}$	0.23	0.25	0.50			0.20	0.16	0.31		

Table 17 : Effect of organic matter application on the effectivity of biofertilizer (N_2-fixer) in managing nitrogen nutrition in lowland kharif rice (in New Alluvial Zone)

Treatments	Straw yield (t ha^{-1})					Grain yield (t ha^{-1})				
	N_0	N_{30}	N_{60}	N_{90}	Mean	N_0	N_{30}	N_{60}	N_{90}	Mean
FYM - applied once/year	3.17	3.75	3.88	3.85	3.66	3.21	3.81	4.00	4.04	3.77
FYM- applied twice/year	3.79	3.89	4.21	4.08	3.99	3.96	4.04	4.10	4.15	4.06
FYM-applied in every alternate year	3.23	3.88	3.88	3.94	3.73	3.10	3.79	4.02	4.10	3.76
FYM- Not applied	3.17	3.37	3.83	3.97	3.58	3.35	3.85	3.83	4.02	3.77
Mean	3.34	3.72	3.95	3.96		3.41	3.87	3.99	4.08	
	FYM	N	FYM x N			FYM	N	FYM x N		
S.Em ±	0.05	0.05	0.10			0.07	0.05	0.10		
$CD_{0.05}$	0.14	0.14	0.29			0.22	0.14	0.29		

Table 18 : Effect organic matter application on the effectiveness of biofertilizer (N_2-fixer) in managing nitrogen nutrition in boro rice (in coastal saline zone).

Treatments	Number of tiller/plant			Straw yield (t ha^{-1})			Grain yield (t ha^{-1})		
	T_1	T_2	Mean	T_1	T_2	Mean	T_1	T_2	Mean
N_{70}	5.53	4.66	5.10	6.5	6.42	6.46	4.16	3.66	3.92
N_{80}	6.47	5.33	5.90	7.25	6.67	6.96	4.75	4.25	4.50
N_{90}	7.20	5.87	6.33	7.58	7.25	7.41	5.16	4.75	4.96
N_{100}	9.00	6.60	7.80	8.08	7.58	7.83	5.66	5.33	5.50
Mean	7.05	5.61	-	7.35	6.98	-	4.93	4.50	-
	FYM	N	FYM x N	FYM	N	FYM x N	FYM	N	FYM x N
S.Em ±	0.96	0.18	0.25	0.11	0.19	0.27	0.18	0.14	0.19
$CD_{0.05}$	NS	0.55	0.77	NS	0.59	NS	NS	0.43	NS

T_1 - FYM @ 5 tha^{-1} ; 'T_2'- no FYM ; 'NS' - not significant.

Table 19 : Effect of biofertilizer and various organic sources on growth and yield attributes of lowland rice (kharif,1996).

Treatment	Plant height* (cm)	Root length* (cm)	Root dry weight* (g)	Shoot* dry weight/hill (g)	Number* of tiller/hill	Number of effective tiller/hill	Panicle length (cm)	Number of grain / Panicle	Grain yield (t ha^{-1})
Control	80.6	17.0	3.5	10.0	16.3	11.3	20.8	180.0	6.46
Rabbit litter	86.0	25.5	3.4	16.7	16.6	13.0	22.5	203.6	8.40
Poultry litter	85.6	20.3	3.8	10.4	22.0	13.6	22.0	201.3	7.26
Sheep litter	85.3	18.3	4.0	13.2	17.3	14.3	23.3	194.3	7.33
Cow dung	80.3	18.1	3.9	14.0	16.3	12.3	20.0	187.0	6.70
S.Em ±	2.95	1.36	0.43	0.73	0.67	0.71	0.56	12.24	0.57
CD $_{0.05}$	NS	NS	NS	2.39	2.19	NS	1.83	NS	NS

* Observations recorded 45 DAT ; 'NS'- Not significant.

was used in conjunction with FYM. However, this benefit appeared to be more pronouncing when FYM was applied twice in a year (i.e. once for each crop season) in comparison to other treatments. It seems quite logical to presume very little residual potentiality of the organic matter applied under a tropical climate like ours. Naturally supplementation of soil with organic matter afresh for each crop would be justified. The present findings also established the potentiality of using diazotrophs in conjunction with organic manure in reducing the inorganic nitrogenous input for rice cultivation to a much greater extent. In this connection, an out standing work made by Roper and Ladha (1995) may be referred, wherein they inoculated straws with cellulolytic and diazotrophic microorganisms to get N-gain to the tune of 72 mg/g of straw consumed. Accordingly, they emphasized improvement in straw associated N_2-fixation in soil by a specific straw management practices which encourage microbial activity by straw decomposing and N_2-fixing microorganisms.

In the present study, there had been practically no difference between the different organic sources applied in boosting the activity of diazotrophs. However, among the different organic sources tested, rabbit litter apparently seem to be superior over others. Incorporation of crop residues, which contain very large amount of carbon, could not be considered in the present study, although it is felt to be incorporated in the future work through the model given by Roper and Ladha (1995).

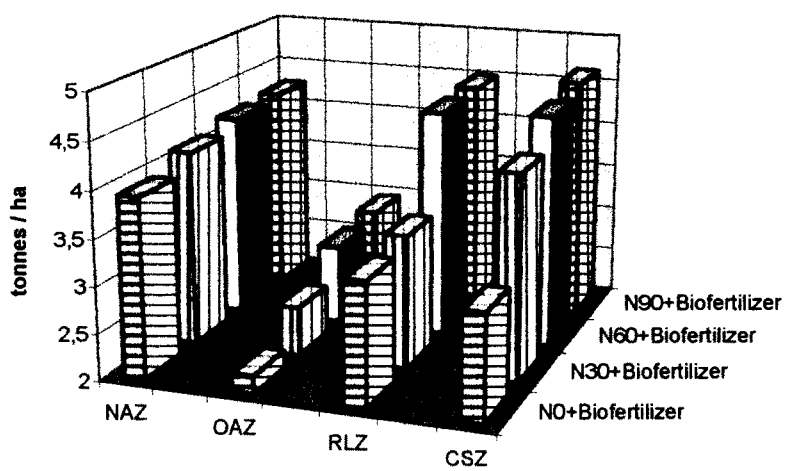
4.8. Performance of biofertilizer under different levels of inorganic nitrogen fertilizer

Inoculation of rice with diazotrophs at different N-levels could bring about variation in respect of plant height, root length, dry weight of shoot

number of tillers/plant and panicle length only (Table-20). All the parameters showed increasing trends with increasing doses of N-fertilizers. This indicated, at least to some extent, that inoculation effect remained operative even at higher level of inorganic nitrogen. Although variation in grain weight due to different treatments was statistically insignificant, the same increasing trend was noticed in higher N-fertilizer. Existence of very little difference in grain yield between highest dose of N-fertilizers (100 kg/ha) and the penultimate dose (80 kg/ha) with inoculation indicated the possibility of curtailing N-fertilizers at least @ 20 kg/ha through effective inoculation schedule.

The above findings encouraged in undertaking certain integrated nutrient management studies by inoculating rice seedlings at different levels of N-fertilizer under field conditions in new Alluvial, Old Alluvial, Red Laterite and Coastal Saline zones. Variation in grain yield of rice in New Alluvial zone appeared to be insignificant, however, results indicated that the yield of rice in this zone at 60kg N-fertilization were almost the same as those obtained at 90kg N-fertilization (Table-21).

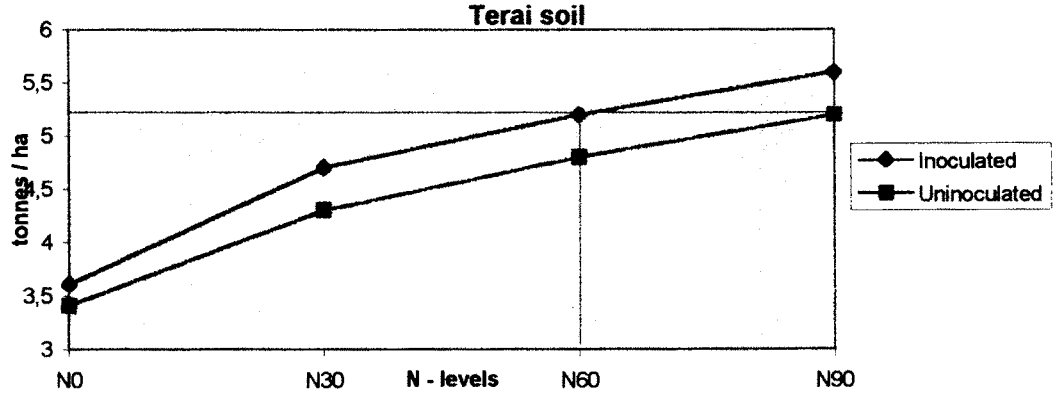
FIG 4: Performance of diazotrophs under different N- regimes in respect of grain yield of rice at three agroecological zones



Similar observations were also recorded in Old Alluvial zone where the grain yield at 60kg and 90kg N along with biofertilizer were not statistically different. Hence, in these two zones, curtailment of N-fertilizer can safety be made to the extent of 30kg/ha under integrated nutrient management practice. Grain and straw yield data obtained from Sekhampur of Red Laterite zone and Kakdwip of coastal saline zone depicted that in both the zone yields at 60 & 90 kg N/ha were statistically same (FIG 4). However, in the later location, the activity of diazotrophs remained much active at lower level of nitrogen and gets reduced at the higher level.

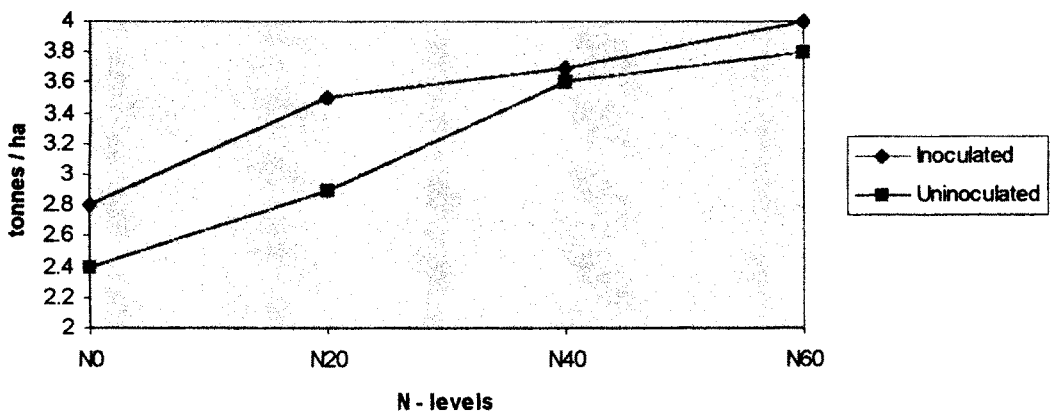
In Terai and Hill zones, the experiments on integrated nutrient management practices were conducted adopting a split plot design keeping the inoculation in main plot. In Terai soil, nitrogen alone could produce significant variations in all the growth and yield parameters of rice (Table-22). It is also observed that inoculation significantly increased grain yield, straw yield and number of tiller over uninoculated control. Results on interactions showed that none of the parameters vary significantly when inoculum interacts with nitrogen. However, for grain yield it was observed that 60kg N/ha along with inoculum could produce almost same yield obtained with 90kg N/ha alone (FIG 5).

FIG 5 : Response of inoculation under different N - regimes in Terai soil



In Hill soil, too, all the parameters vary significantly due to the effect of nitrogen. However, inoculation could bring about significant variation in number of tillers and straw weight (Table-23). Interaction was significant only for straw weight. It appears that inoculation could significantly increase straw yield when interact with 0 to 40 kgN ha^{-1} and above this N-level, variation was insignificant. Observations also revealed that straw yield obtained with 40KgN ha^{-1} along with inoculum was statistically similar with that obtained with 60 kg N/ha alone. Grain yield did not vary significantly , however response of inoculation was very high at lower N - levels (FIG 6).

FIG 6 : Response of inoculation under different N - regimes in hill soil



Earlier experiment (Rangaswami, 1975) also reported that nitrogen requirement of paddy could be reduced by 20 to 40 kg N/ha by *Azotobacter* inoculation. Gopalaswami *et al.* (1989) showed that *A. lipoferum* inoculation to soil fertilized with 75 kgN/ha significantly increased the grain yield. But at lower (25, 50kg N/ha) or higher (100 kg N/ha) N-level, the bacterial inoculation did not have any significant beneficial effect. The present study

Table 20 : Performance of biofertilizer under different levels of inorganic nitrogen fertilizer in lowland rice field in New Alluvial zone (kharif, 1996).

Treatment	Plant height* (cm)	Root length* (cm)	Root dry weight* (g)	Shoot dry weight* (g)	Number* of tiller	Number of effective tiller	Panicle length (cm)	Number of grain / Panicle	Grain yield (t ha^{-1})
N_{60}	73.3	22.0	4.9	14.8	20.0	10.3	19.5	170.3	6.6
N_{60}+Inoculation	77.3	23.5	4.2	12.9	21.0	11.0	21.2	186.0	6.9
N_{80}	77.6	22.6	4.4	16.8	22.3	12.3	20.2	179.3	8.0
N_{80}+Inculation	85.3	23.5	5.2	20.1	24.3	13.0	22.2	191.3	8.6
N_{100}	79.3	23.5	5.6	18.1	24.0	11.6	20.3	186.0	8.7
N_{100}+Inoculation	86.3	24.6	6.1	24.7	26.3	14.6	23.2	201.0	8.3
S.Em±	1.87	0.41	0.63	2.09	1.16	1.01	0.54	11.13	0.56
CD 0.05	5.30	1.31	NS	6.59	3.68	NS	1.69	NS	NS

* Observations were recorded 45 days after transplantation; NS-not significant.

Table 21 : Performance of composite diazotrophic inoculum under different nitrogen regime in lowland rice field in four agro-ecological zones of West Bengal (Kharif,1997)

Treatments	Grain yield (t / ha)				Straw yield (t / ha)		
	New Alluvial Zone	Old Alluvial Zone	Red Laterite Zone	Coastal Saline Zone	New Alluvial Zone	Red Laterite Zone	Coastal Saline Zone
N_0 + biofertilizer	3.92	2.19	3.31	3.13	3.67	2.75	4.26
N_{30} + biofertilizer	4.10	2.53	3.43	4.19	3.77	2.80	6.14
N_{60} + biofertilizer	4.21	2.85	4.45	4.50	3.98	2.88	6.42
N_{90} + biofertilizer	4.25	2.94	4.49	4.63	4.25	3.13	6.98
S. Em ±	0.13	0.09	0.07	0.12	0.09	0.07	0.24
$CD_{0.05}$	NS	0.31	0.25	0.39	0.30	0.25	0.76

Table 22 : Response of lowland rice to mixed inocula (N_2-fixing) under different nitrogen regime in Terai soil. (Kharf, 1997).

Treatment	Plant height (cm)			Effective tiller			Panicle length (cm)			Numbers of grain/Panicle			1000 Grain weight (g)			Straw yield (t ha^{-1})			Grain yield (t ha^{-1})		
	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean
N_0	69.3	70.7	70.0	9.9	10.0	9.9	17.5	17.2	17.3	77.8	78.1	78.0	18.1	17.8	17.9	2.5	2.8	2.6	3.4	3.6	3.5
N_{30}	73.3	73.5	73.4	12.8	13.1	12.9	18.8	18.9	18.8	83.3	84.1	83.7	19.1	18.8	18.9	3.0	3.5	3.2	4.3	4.7	4.5
N_{60}	74.3	74.4	74.4	13.4	14.0	13.7	18.8	19.4	19.1	85.0	86.0	85.5	20.2	20.3	20.3	3.4	3.7	3.6	4.8	5.2	5.0
N_{90}	76.4	77.1	76.7	14.5	14.9	14.7	19.9	19.9	19.9	89.2	90.5	89.9	21.0	21.4	21.2	3.6	3.9	3.7	5.2	5.6	5.4
Mean	73.3	73.9	-	12.6	13.0	-	18.7	18.8	-	83.8	84.7	-	19.6	19.6	-	3.1	3.5	-	4.4	4.8	-
	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN
SEm±	0.18	0.52	0.74	0.06	0.13	0.18	0.17	0.22	0.32	0.32	0.59	0.84	0.15	0.25	0.36	0.07	0.06	0.08	0.03	0.07	0.11
CD0.05	NS	1.54	NS	0.27	0.38	NS	NS	0.66	NS	NS	1.75	NS	NS	0.74	NS	0.32	0.18	NS	0.15	0.22	NS

I_0 - Ininoculated ; I_1 - Inoculated ; NS - Not significant.

Table 23 : Effect of diazotrophs on various growth and yield attributes of rice under different N regime in Hill soil (kharif, 1997)

Treatment	Plant height (cm)			Effective Tiller			Panicle length (cm)			Straw yield (t ha^{-1})			Grain yield (t ha^{-1})		
	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean	I_0	I_1	Mean
N_0	100.4	102.8	101.6	6.4	7.5	7.0	19.4	20.3	19.9	3.2	3..8	3.5	2.4	2.8	2.6
N_{20}	102.9	104.8	103.85	7.1	8.0	7.6	21.0	21.6	21.3	3.5	4.1	3.8	2.9	3.5	3.2
N_{40}	105.0	105.2	105.1	7.9	8.4	8.2	21.8	22.2	22.0	3.9	4.4	4.1	3.6	3.7	3.7
N_{60}	107.0	106.9	106.95	8.3	8.6	8.5	22.4	22.8	22.6	4.3	4.4	4.3	3.8	4.0	3.9
Mean	103.8	104.9	-	7.4	8.1	-	21.2	21.7	-	3.7	4.2	-	3.2	3.5	-
	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN	I	N	IxN
SEm±	0.44	0.97	1.37	0.12	0.22	0.30	0.26	0.44	0.63	0.05	0.05	0.06	0.07	0.15	0.21
$CD_{0.05}$	NS	2.88	NS	0.54	0.64	NS	NS	1.33	NS	0.24	0.14	0.19	NS	0.44	NS

I_0 - Uninoculated ; I_1 - Inoculated ; NS - Not significant

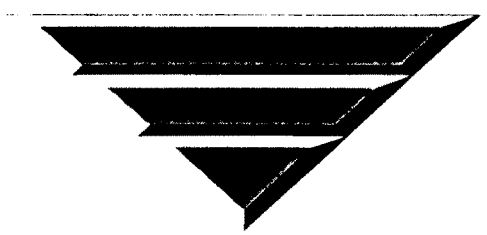
also depicted the beneficial effect of inoculum in the similar N-level. Heulin *et al.* (1991) conducted inoculation experiment in Egypt and Bangladesh with *Azospirillum* sp. and observed that A. *brasilense* had a significant positive effect on grain yield in all Egyptian soil, conversely, in the Bangladesh experiment, where nitrogen fertilizer had no effect on yield, *A. lipoferum* inoculation was effective in spite of the adaptation of this bacterium to the rhizosphere of rice. In the present study by using same inoculum for Terai and Hill zone, the significant increase of grain yield was observed only in Terai soil, however, in both the cases, N alone can brought about significant variation in grain yield.

Table 24 : Comparison of input cost for rice cultivation under conventional practice and under integrated plant nutrient management system (Kharif rice)

Requirements of inputs (per ha)		Cost of inputs (in Rs)	
Conventional system	IPNS	Conventional system	IPNS
FYM 2t	FYM 2t	Rs.500.00	Rs.500.00
N 80 kg	N 60 kg	Rs.713.00	Rs.534.00
P_2O_5 40 kg	P_2O_5 40 kg	Rs. 750.00	Rs.750.00
K_2O 40 kg	K_2O 40 kg	Rs.266.00	Rs.266.00
Biofertilizer - Nil	Biofertilizer- 2.5 kg	-	Rs.50.00
Total		Rs.2229.00	Rs.2100.00

From the above findings it becomes clear that while practicing integrated nutrient management for rice cultivation, it should be borne in mind that the optimum amount of inorganic inputs to be applied would depend much on the type of agricultural Zone, soil characteristics and the strain of diazotrophs used. Proper monitoring under such package of practices would be the most vital aspect since this must ensure minimum wastage of inputs vis-a-vis least reduction in the optimum crop yield. While comparing the input cost (Table-24), it indicates that integrated plant nutrient management system can save negligible amount in terms of money over conventional system when producing the same yield. However, it would not be wise to evaluate these two system in terms of money only as because the integrated plant nutrient management system is much worthy in restoration of soil health, sustainability of crop production and reduction in pollution.

CHAPTER - V



SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

The root region of rice plant is a niche of intense microbial activity. The microorganisms in rhizosphere are not only numerically higher but also physiologically more efficient. However, the number of specific organism around root depends on selective stimulation/suppression by the host plant depending upon its growth stages and composition of the root exudates. Such stimulation may be aimed at enhancing proliferation of different plant growth promoting rhizobacteria and rhizocoenotic diazotrophs.

Considering the above back ground knowledge, many workers conducted inoculation experiments with efficient bacterial strains but inoculation always did not result in consistent yield responses owing to different ecological factors, and varying environmental conditions. Hence, in the present study , attempts were made to select efficient diazotrophic strains in different agroecological zones, to study the effect of organic matter and graded N-level on the performance of diazotrophs. Experiments were also conducted on the methods of inoculation to make the inoculation effect more pronounced. The findings of different experiments are presented below.

Predominant colonies were isolated by using specific media used for *Azotobacter, Azospirillum, Bacillus, Derxia, Klebsiella* and *Pseudomonas.* None of the isolates obtained from media for *Klebsiella, Bacillus* and *Alkaligenes* showed positive ARA. However, 77.7% *Azotobacter*, 72.2% *Derxia* , 50% *Azospirillum* and 25% *Pseudomonas* like organisms showed acetylene reduction activity. Among the isolates, maximum ARA was observed by *Pseudomonas* followed by *Derxia, Azotobacter* and *Azospirillum.* When N_2-fixing capacity was measured in

respect of per gram sugar utilization, *Derxia* like organisms were found to fixed maximum atmospheric nitrogen followed by *Pseudomonas, Azotobacter* and *Azospirillum.* Moreover, none of the isolates fix more than 10mg Ng^{-1} of carbon source. Another screening experiment showed that among the thirty three diazotrophs, only eight produced IAA.

During characterization, it was observed that isolates A6, A7, P12 were easily matched with the character of *Azotobacter, beijerinckia, Azotobacter chroococcum* and *Pseudomonas,* respectively whereas, Ap3 and Ap18 both showed character like *Azospirillum lipoferum.* However, morphological, cultural and biochemical characters of D2, P3 and P4 did not give enough information to match with known diazotrophs according to Bergey's Manual of Systematic Bacteriology.

Interactions between three rice cultivars and eight diazotrophs showed that irrespective of rice varieties, *Azospirillum* has emerged out as the best inoculum followed by *Pseudomonas*(P12) and *Azotobacter*(A7). Among the 'Variety X diazotroph' combinations, *Azospirillum, Pseudomonas and Azotobacter* appeared to be the best when inoculated to rice variety IR-50. In the present study, the isolates D2, P3 and P4 have failed to produce any encouraging result although the same have showed better ARA under culture condition. On the contrary, Ap18 showed better result in spite of having low ARA.

Performance of eight selected diazotrophs varies considerably in different agricultural zones of West Bengal. In Terai zone, pot experiment showed that grain yield scored better when inoculated with *Azospirillum*(Ap18). In Red Laterite zone and Coastal Saline zone, maximum response in respect of grain and straw yield was exhibited by an isolate of *Pseudomonas* like organism(P4). Two sets of isolates evaluated

in new Alluvial zone, depicted that grain yield of rice was maximum with *Derxia* (D2) and *Azotobacter* (A9) like organisms.

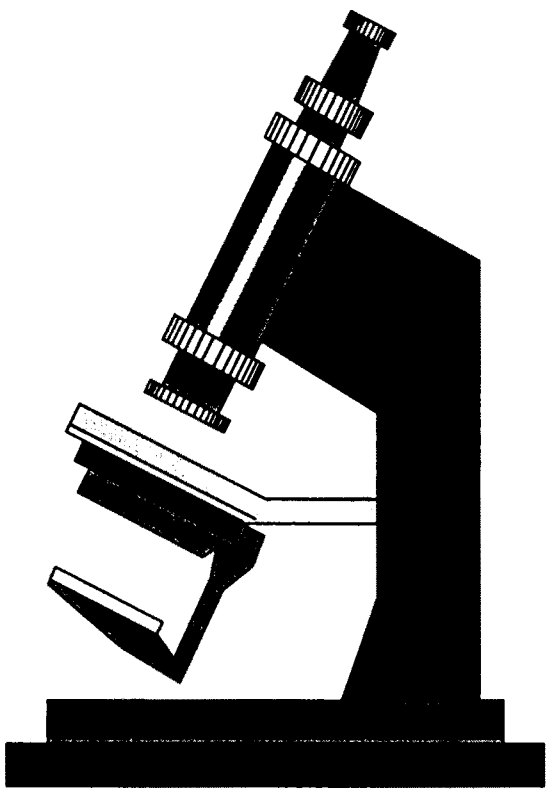
Experiment was conducted to assess the effectiveness of different inoculation methods and among the three methods of inoculation seedling dipping appeared to be better than seed bed and seed inoculation. In another experiment, single and mixed inocula were compared. It was observed that all the mixed inocula showed better performance as compared to single inoculum and 'Ap18+A7' combination was found to be the superior among diferent mixed inocula. It can also be highlighted that mixed inocula comprising of aerobic and microaerophilic diazotrophs had better performance.

Performance of diazotrophs under different FYM enrichment levels were studied in New Alluvial and Coastal Saline zone. It was observed that in New Alluvial zone maximum grain and straw yield were recorded when biofertilizer was applied in soil enriched with FYM twice in a year i.e. before each crop cycle in a year @ 2 t ha^{-1}. But in coastal Saline zone effect of FYM did not vary significantly. However, in each treatment, FYM @ 5 t ha^{-1} increase number of tiller, grain and straw yield over control. In a different experiment, performance of biofertilizer was evaluated under different organic sources and it was observed that the effect of cow dung, rabbit litter, poultry and ship litter did not vary significantly.

Integrated nutrient management studies showed in New Alluvial and Old Alluvial zone the grain yield obtained at 60 kg and 90 kg N ha^{-1} are statistically same. In Red Laterite and Coastal Saline zone also, yield at 60 and 90 kg N/ha are did not vary significantly. Hence, for these zones curtailment of nitrogen can safely be made to the extent of 30 kg N ha^{-1}. Similar recommendation can also be made for Terai zone, too.

In conclusion it can be highlighted that while selecting diazotrophs strains as ideal rice inoculant, emphasis should be given more on using region specific best adapted strains than their laboratory performance. The proliferation and activity of a diazotroph are much dependent on different environmental factors of a particular ecological situation to which the strain is exposed. Hence, none of the effective strains of diazotrophs can be recommended for universal application unless it is tested against a particular crop under a particular agroecological situation. Integrated nutrient management system including biofertilizer as a component helped in curtailing input costs to some extent that may not appear to be much economic in terms of money. But if it is considered from different angles like restoration of soil health, bringing about sustainability in crop production, reduction in the pollution problems etc., then the system would proved worthy.

CHAPTER - VI



FUTURE SCOPE OF RESEARCH

FUTURE SCOPE OF RESEARCH

During the course of present study some new avenues of research are identified about the biological nitrogen fixation associated with rice. Moreover, a number of questions remain yet to be answered for better understanding of the subject and to develop an effective N_2-fixing system in rice root zone. Few of these ideas are discussed below.

Several experiments provide information that the extent of effect in terms of growth promotion and N-uptake due to inoculation with diazotrophs can not be explained well with the amount of nitrogen fixed through BNF. Therefore, several authors refer to the production of plant growth regulators to explain the beneficial effect of bacterial inoculation. So, work can be undertaken to understand and verify the role of inoculated rhizobacteria that produce PGRs.

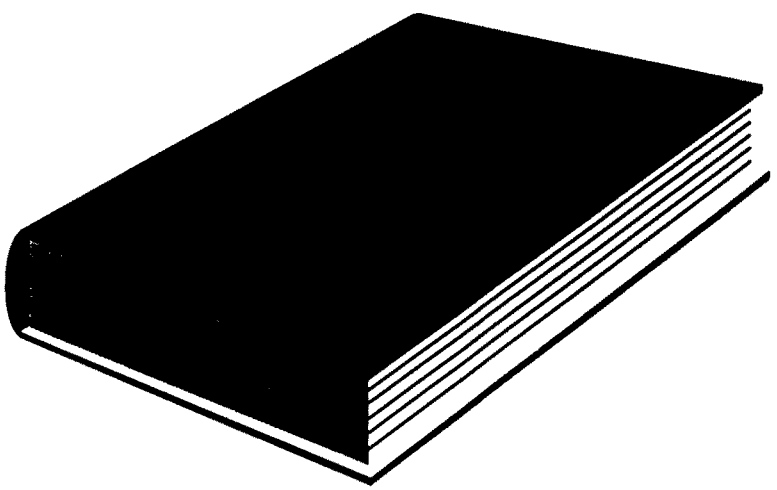
Little information is available about the establishment of inoculated strain and competition between indigenous and inoculated strain. Hence, it becomes difficult to explain the inoculation effect without knowing the establishment of inoculated bacteria. So, more research is required to study the fate of inoculated bacteria with the help of marker loaded strains.

More effect should be given to isolate efficient diazotrophs from different agro-ecological zones. Establishment potentiality and antagonistic properties towards other rhizospheric bacteria must be considered in screening programme.

Recent advances in understanding symbiotic *Rhizobium*-legume interactions at the molecular level and the ability to introduce new genes into rice by transformation have created an excellent opportunity to

investigate the possibilities for incorporating N_2 fixation capacity in rice. Exploratory research is needed to assess the feasibility of non-nodular or endophytic associations, nodular associations and *'nif'* genes transfer to rice plant.

CHAPTER - VII



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