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Nitrogen fixation by free-living microorganisms in tropical rice soils using labelled fertilizer, (part of a coord.progr. on isotope techniques in studies of biological nitrogen fixation for the dual purpose of increasing crop production and decreasing nitrogen fertilizer use to conserve the environment)

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FINAL REPORT

Project No. RC/1804/R-4/SD on "Nitrogen fixation by free-living microorganisms in rice soils".

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ABSTRACT

Heterotrophic nitrogen fixation as influenced by the water regime, organic matter, combined nitrogen, pesticides, cultural practices and plant genotype was investigated in several Indian rice soils employing $^{15}N_2$ tracer and gas chromatographic acetylene reduction techniques. Soil submergence accelerated nitrogen fixation. Addition of cellulose, rice straw to both flooded and nonflooded soils enhanced nitrogen fixation. Under submerged conditions, addition of sucrose, glucose and malate in that order stimulated nitrogen fixation in alluvial soil, while only sucrose enhanced nitrogen fixation in laterite soil. However, in acid sulphate and acid saline soils, the addition of either indigenous or synthetic organic carbon source was not effective in enhancing nitrogen fixation. Nitrogen fixation decreased with increasing concentrations of ammonium sulphate; under nonflooded conditions, this inhibition was more pronounced. Nitrogen fixation was completely inhibited by ammonium sulphate in acid sulphate and saline soils.

Application of certain pesticides at rates almost equivalent to recommended field levels, exerted profound influence on nitrogen fixation in submerged rice soils. Additions of benomyl (carbamate fungicide) and carbofuran (methyl carbamate insecticide) to alluvial and laterite soils resulted in a significant stimulation of nitrogen fixation. Gamma-BHH, a chlorinated hydrocarbon insecticide, stimulated nitrogen fixation in only alluvial soil, while considerable inhibition was evident in a laterite soil. Results also showed differential responses of specific groups of nitrogen-fixing organisms to the pesticides depending upon the soil type. A synergistic stimulatory effect of certain pesticide combinations on nitrogen fixation

was observed. Certain natural and synthetic compounds having insecticidal activity inhibited the rhizosphere nitrogenase activity.

Nitrogen fixation by Azospirillum lipoferum isolated from several rice cultivars was investigated by $^{15}N_2$ incorporation. Large variations in $^{15}N_2$ incorporation by A.lipoferum isolated from the roots of several rice cultivars were observed. The possibility for selecting/breeding the specific lines of rice harbouring A.lipoferum with high nitrogenase activity is suggested. Striking stimulation of Azospirillum population occurred in rice straw and benlate-amended field soils. Azospirillum isolates obtained from various rice field weeds were more efficient than that from the phyllosphere in their nitrogen-fixing efficiency. An acid-sulphate saline soil of extremely low pH (3.2) harboured Azospirillum spp. with appreciable nitrogen-fixing activity. Enrichment cultures originating from soils with low pH (< 4.0) possessed lower nitrogen-fixing activity compared to cultures from soils with higher pH values (upto 6.6). Azospirillum cultures from soils that had undergone prolonged waterlogging showed lower nitrogen-fixing activity than cultures isolated from soils submerged for a few days. A relationship was shown between the in vitro nitrogen-fixing activity of Azospirillum cultures and the redox status of the soil samples; activity was high when the soil redox potential was between -50 to -150 mV. The results show that the nitrogen-fixing activity of Azospirillum cultures is governed by fluctuations in soil redox potential, pH and organic matter.

The rhizosphere soil samples from different rice cultivars showed striking differences with regard to their ability to incorporate $^{15}N_2$. Moreover, rhizosphere samples from rice straw-amended (3 and 6 tons/ha) soil exhibited more pronounced nitrogen-fixing activity than the samples from unamended soils, while the activity of the rhizosphere samples from soils receiving combined nitrogen (40 and 80 kg N/ha) was relatively low. However, the inhibitory effect of combined nitrogen was not expressed in the presence of increased levels of rice straw (6 tons/ha).

The exact quantitative estimate of the amount of nitrogen fixed by heterotrophic organisms in a complex system such as submerged soil, has not been possible hitherto. Circumstantial evidence from this study and elsewhere suggests that indigenous nitrogen fixation in a flooded soil would be in the range of 2-10 kg N/ha; and this could be accentuated following judicious

amendments with organic and mineral fertilisers and cultural practices. These studies also indicate the implication of certain management practices for efficient nitrogen fixation in rice systems.

INTRODUCTION

Fixed nitrogen is often the major limiting factor in the crop production. Nitrogen fixation under rice cultivation is of great significance since the conditions prevailing in lowland soils favour nitrogen fixation. Moreover, rice soils offer unique conditions for the activity of free-living heterotrophic nitrogen-fixing bacteria by virtue of low oxygen pressure and adequate moisture and nutrient availability. Although flooded rice soils have greater nitrogen accumulating power compared to other upland soils, no clear-cut evidence on the magnitude of nitrogen fixation is available. The first report came from International Rice Research Institute, Philippines employing ^{15}N-tracer technique. Compared to nonflooded soils, flooded soils, in general, exhibit greater nitrogen-fixing activity irrespective of the presence of rice plant. Moreover, soil submergence even for a short period, accelerated nitrogen fixation. It has also been established that the nitrogen-fixing activity in flooded soils could be further enhanced by the addition of rice straw, rice roots and other related energy-rich materials. Rice plant is known to stimulate nitrogen fixation in flooded soils and the nitrogen-fixing activity is intense particularly during maximum tillering-flowering stages of the rice plant. Moreover, for heterotrophic nitrogen fixation readily available carbon source is the major limiting factor in most of the paddy soils and substantial gains in nitrogen fixation were noticed following the addition of several carbon substrates.

The role of plant bacteria associations in the nitrogen economy has been well established in several grass and cereal crops. The occurrence of Azospirillum spp. in rice roots and paddy soils needs to be investigated. This opens up the possibility of a significant contribution by this group of associative microorganisms with several higher plants.

Studies were initiated at this Institute to follow the magnitude of nitrogen fixation in rice soils and factors affecting this process employing ^{15}N-tracer and acetylene reduction techniques. Asymbiotic nitrogen fixation as influenced by moisture regime organic sources and pesticides in several

Indian rice soils was investigated. Studies were also conducted on the soil-plant-microbe interaction with special reference to nitrogen-fixing Azospirillum sp.

MATERIALS AND METHODS

Soils collected from different rice growing tracts of India belonging to alluvial, laterite, acid sulphate saline and acid saline types were used in the present study. Some properties of the soils are described in Table 1.

The soils were air-dried, screened (2 mm), and placed in glass vials (1.2 cm x 5.0 cm) in 5-g amounts. All the soils were amended with 0.5 or 1.0 per cent powdered cellulose and thoroughly mixed with the soil prior to flooding. One set of the soils was flooded (1.5 cm standing water column), and the other was held nonflooded (50% WHC). All the treatments were replicated thrice.

In addition to continuous flooded and nonflooded conditions, several alternate flooded and nonflooded conditions were maintained for different periods during a 48-day incubation. At periodic intervals nitrogenase was assayed employing gas chromatographic C_2H_2 reduction assay. Water was added to a nonflooded soil, upto a 1.7 cm column, to provide submerged condition. To achieve the nonflooded (drained) condition in a submerged soil, the water column above the soil was decanted. After the flood water was removed, the moisture content of the soil was determined separately. The moisture content reached 20 and 30% in alluvial and Pokkali soils, respectively, within 4 to 6 days after draining, corresponding to the moisture content of a nonflooded sytem. At periodic intervals, water was added to the soils in all treatments to compensate for the evaporation loss. C_2H_2 reduction was negligible in decanted water from a flooded soil.

To study the effect of N fertilization on nitrogen fixation, 5-g portion of the soils amended with 1.0 per cent cellulose, were mixed respectively with $(NH_4)_2SO_4$ corresponding to 20, 40, 60, 80 and 100 ppm N in the vials. These were incubated under both flooded and nonflooded conditions.

The pesticides used in the study consisted of two insecticides carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) and gamma-BHC (gamma-1,2,3,4,5,6-hexachlorocyclohexane) and a fungicide benomyl (methyl-1-(butyl-carbamoyl)-2-benzimidazole-carbamate). Technical grade pesticide formulations were applied to the soils at 5 parts/10^6 in acetone prior to flooding. Control treatments received only acetone.

In pot experiments 20-day old seedlings (cv. Supriya) were transplanted and maintained till maturity. Three levels of combined nitrogen as ammonium sulphate at 0, 40 and 80 kg N per ha were applied in two splits at transplanting and maximum tillering stages. Another set of the pots received two levels of rice straw at 3 and 6 tons per ha level. Rice straw was applied to the flooded soil 30 days before transplantation and allowed to decompose. In the third set the rice straw amended (3 and 6 tons/ha) pots received combined nitrogen at 0, 40 and 80 kg N/ha. Unplanted pots served as nonrhizosphere controls. After 60 days the rhizosphere soil was collected from all the treatments and the soil samples were transferred to glass vials for $^{15}N_2$ incorporation studies.

Fifteen day old seedlings of twenty high yielding semi-dwarf rice cultivars were transplanted and were grown under uniform field conditions in three replicate microplots under submerged conditions without fertilizer application. After 60 days of transplanting, the rhizosphere soil was collected and the samples from each variety were placed in glass vials and amended with 0.5% cellulose. The samples were incubated in an $^{15}N_2$ atmosphere.

A field experiment for the evaluation of relative efficiency of synthetic and natural compounds having insecticidal activity was conducted. Two organophosphate insecticides quinalphos and phosalone at 0.5 kg/ha and plant products pyrethrum, neem extract, neem oil and allitin were sprayed on the plants on 50, 60 and 75 days after transplantation. Six plants per each treatment were carefully uprooted after 10 days of the application of the respective compounds and the rhizosphere samples were transferred to vacutainer tubes for acetylene reduction analysis.

The influence of water regime, combined nitrogen and pesticides on nitrogen fixation in soils was investigated by employing $^{15}N_2$ isotope method. Soil samples amended with cellulose (0.5-1.0%) and different levels of combined nitrogen and pesticide formulations were transferred to a desiccator

wrapped with black paper to prevent algal growth. Excess carbon dioxide produced during the incubation of the soils was absorbed by a 40% KOH solution placed in the desiccator at the beginning of the experiment. The desiccator was then sealed and the air was evacuated by vacuum and flushed three times with argon. An atmosphere containing 72.5 atom% excess $^{15}N_2$ (Prochem, London), which consisted of oxygen (0.20 atm), argon (0.50 atm) and nitrogen (0.30 atm $^{14}N_2 + {}^{15}N_2$) was introduced into the desiccator. The system was then incubated at 28°C ± 2°C for 28 days in dark. Fresh portions of O_2 were added (2 times a week) to compensate for the consumed O_2. At the end of the incubation period, the total N in the soil samples (triplicate) was determined by the Kjeldahl method and the distillates were used for the determination of ^{15}N enrichment of the soils. The NH_3 distilled from soil extracts were absorbed in HCl (0.1 N) and back titrated with standard NaOH (0.1 N). After acidification, the above samples were oven-dried at 70°C. The isotopic ratio analysis of the samples was conducted on a mass-spectrometer.

Nitrogen-fixing microorganisms were isolated from different soils following conventional microbiological techniques on N-free liquid and solid media. In view of the potential participation of Azospirillum sp. in nitrogen fixation in several ecosystems, concentrated efforts were made on this organism in the present study. The semi-solid malate medium was used for the isolation of Azospirillum from rice roots and rice soils. A.lipoferum was isolated from the surface sterilized roots of several rice cultivars grown under uniform field conditions. The organisms from rice soils and the roots of several rice cultivars possessed the same characteristics and belonged to Azospirillum lipoferum (Beijerinck) Comb. nov. (Group II). $^{15}N_2$ incorporation by A.lipoferum was investigated by exposing the cultures in 30 ml semi-solid nitrogen-free malate medium contained in special 100 ml vacuum flasks for 4 days. In addition, organisms belonging to Azotobacter, Clostridium, Bacillus polymyxa, Pseudomonas sp. and nitrogen-fixing symbiotrophic associations were also isolated from these soils and their participation in nitrogen fixation was investigated.

RESULTS AND DISCUSSION

The data on the isotopic analysis of the soil nitrogen indicated appreciable nitrogen-fixing activity even in the absence of organic amendment in all the four soils studied (Table 2). Greater nitrogen-fixing activity was observed in submerged laterite and alluvial soils. Despite high acidity and salinity significant nitrogen fixation occurred in pokkali and kaarapadam soils under both water regimes. Soil submergence increased nitrogen fixation irrespective of the soil type. The low nitrogen-fixing activity under nonflooded

conditions may be a consequence of high O_2 tension, which has been claimed to inhibit nitrogen fixation and limiting water content. Greater nitrogen fixation under flooded conditions has been attributed to the adequate moisture content, nutrient supply and favourable conditions for the activity of nitrogen-fixing microorganisms.

Application of organic matter (cellulose) stimulated nitrogen fixation under both flooded and nonflooded conditions. Similar gains in nitrogen fixation with the addition of organic sources were observed in several soils. It has been established that under flooded conditions, products of anaerobic decomposition of organic material may serve as energy sources for aerobic, facultative anaerobic nitrogen fixers. Addition of cellulose and rice straw, in general, stimulated nitrogen fixation in flooded alluvial, laterite and acid sulphate (pokkali) soils (Table 3). Cellulose was superior to rice straw as an energy source for nitrogen fixation under both flooded and nonflooded conditions in alluvial and laterite soils. However, in pokkali soil rice straw was more effective in enhancing nitrogen fixation under both water regimes.

Under submerged conditions addition of sucrose, glucose and malate in that order stimulated nitrogen fixation in alluvial soil, while sucrose alone stimulated nitrogen fixation in laterite soil. However, in submerged acid sulphate soil the above three sources did not exhibit any influence on nitrogen fixation. Nitrogen fixation was greater in cellulose-, sucrose- and glucose-amended alluvial soil under nonflooded conditions. Malate addition stimulated nitrogen fixation in only submerged alluvial soil. Nonflooded conditions in pokkali soil supported nitrogen fixation while this was negligible under submerged conditions. It is interesting to note that in alluvial soil nitrogen fixation was significantly higher under nonflooded than under submerged conditions. This striking difference could perhaps be due to greater invertase activity in nonflooded alluvial soil leading to increased glucose availability. Studies at this laboratory (Chendrayan and Sethunathan, unpublished data) have already shown the higher invertase activity under nonflooded conditions in this soil. Evidently, higher amounts of glucose under nonflooded conditions possibly led to the increased nitrogen fixation. Moreover, greater nitrogen fixation occurred in glucose-amended alluvial soil incubated under nonflooded conditions.

The influence of combined nitrogen on nitrogen fixation in soils under flooded and nonflooded conditions was investigated. Studies indicate that

increased levels of NH_4^+-N decreased nitrogen fixation in alluvial soil under both flooded and nonflooded conditions (Table 4). Moreover, the inhibition is more pronounced under nonflooded conditions beyond 60 ppm N. The inhibitory effect of added nitrogen is more drastic in a laterite soil particularly under nonflooded conditions where even 20 ppm N completely suppressed nitrogen fixation (Table 5). Although the inhibitory effect of combined nitrogen is marked and drastic, appreciable nitrogen fixation occurred at 40 ppm N in submerged laterite soil. Contrary to this, in acid sulphate (pokkali) soil, nitrogen fixation was completely suppressed in the presence of combined nitrogen under both water regimes (Table 6). These results suggest that the nature of inhibition of nitrogen fixation by combined nitrogen depends on the soil type, water regime and the concentration of the combined nitrogen. Complete suppression of nitrogen fixation in acid sulphate soil irrespective of the quantity of the combined nitrogen indicates that in this soil the nitrogen-fixing heterotrophic organisms are more sensitive to combined nitrogen than the organisms in alluvial and laterite soils where a gradual decrease in the nitrogenase activity was evident.

Nitrogen fixation as influenced by the alternate flooded and nonflooded conditions in two paddy soils amended with cellulose was investigated employing gas chromatographic acetylene reduction assay (Table 7 and 8). The soil nitrogenase activity was higher under submerged conditions compared to nonflooded conditions (Table 7). The alternate flooded and nonflooded regimes resulted in alterations of the nitrogenase activity. Thus, nitrogenase activity increased several fold following a shift from nonflooded to flooded conditions. In contrast, the nitrogenase activity decreased when the flooded soil was returned to nonflooded conditions by draining. Moreover, soil submergence favoured nitrogen fixation irrespective of the length of the pre-incubation under nonflooded conditions. Similar trend on the influence of alternate flooded and nonflooded conditions on nitrogenase was observed in an acid sulphate pokkali soil (Table 8). These results are of particular relevance in rainfed rice soils as the occurrence of dry and wet conditions is common as a consequence of drainage and evaporation.

The effect of a chlorinated hydrocarbon and two carbamate pesticides at recommended field application rates on heterotrophic nitrogen fixation in organic amended submerged paddy soils was investigated. Benomyl, a carbamate fungicide, exhibited marked stimulation on nitrogen fixation in alluvial and

laterite soils (Table 9). Striking stimulation in the population and nitrogen fixation by a nitrogen-fixing Azospirillum sp. was observed in benomyl-amended paddy soil. Carbofuran, a methyl carbamate insecticide, significantly enhanced nitrogen fixation in an alluvial soil, while slight stimulation was noticed in laterite soil. Interestingly, carbofuran had no effect on nitrogen fixation in an acid sulphate saline pokkali soil. Studies from our laboratory on the persistance of carbofuran indicated that this insecticide was more persistent in pokkali than in laterite and alluvial soils. The greater persistence of carbofuran in acid soils and unfavourable pH and soil conditions might have resulted in the almost complete suppression of nitrogen fixation. Evidently, in soils where rapid degradation of carbofuran occurred greater nitrogen fixation was observed. Depression of nitrification by carbamate compounds in soil systems was noticed and this depended on the soil properties. Our results further indicate that carbamate insecticides exhibit both stimulatory and inhibitory influence on nitrogen fixation depending upon the soil type. Differential response of γ-BHC was noticed depending upon the soil type. Profound stimulation of nitrogen fixation occurred in alluvial soil while in other two soils nitrogen fixation was either inhibited or maintained at the same level.

Microbiological analysis showed that the effects of pesticides on the population of nitrogen-fixing organisms were related to the pesticide used, soil type and specific groups of nitrogen fixers (Table 10). Thus, Azospirillum was stimulated by benomyl in alluvial, laterite, and Karapadam soils, and by carbofuran only in alluvial soil. With respect to anaerobic nitrogen fixers benomyl was stimulatory only in alluvial soil, and carbofuran in alluvial and laterite soils. Azotobacter was not markedly influenced by either pesticide irrespective of the soil used. Clearly, these results demonstrate differential effects of pesticides on specific groups of nitrogen fixers accounting for differences in nitrogen fixation in flooded soils.

Our studies further indicate that certain pesticides like benomyl and carbofuran, exert remarkable influence on nitrogen fixation by heterotrophic bacteria in submerged soils even at recommended field level applications. This emphasises the need to evaluate the proper use of pesticides to a particular soil type having a dual role in combating pests and diseases on the one hand and increasing the soil fertility on the other.

Plant genotype plays an important role in determining the rhizosphere microbial characteristics. Although nitrogen-fixing Azospirillum has been isolated from several plant species, data on the genotypic differences with respect to the nitrogen-fixing ability of the Azospirillum isolates within the same plant species are not available. We studied the $^{15}N_2$ incorporation by cultures of Azospirillum lipoferum isolated from several rice cultivars (Table 11). The $^{15}N_2$ incorporation by Azospirillum lipoferum cultures isolated from different rice cultivars, grown under uniform field conditions, using 72.5 atom% $^{15}N_2$ ranged from 11.8 to 0.2. Evidently, isolates from different rice cultivars markedly varied in their ability to incorporate $^{15}N_2$. Since A.lipoferum is very closely associated on or inside the root tissue, the differential nitrogen fixation could be due to qualitative and quantitative differences in the root environment from which these were obtained. It is also possible that certain isolates of A.lipoferum could utilise the available nutrients more efficiently than other isolates with concomitant variations in the nitrogenase activity among the isolates. Evidently, it may be possible to select/breed specific lines of rice that harbour A.lipoferum with high nitrogen-fixing ability.

Nitrogen-fixing Azospirillum spp. were isolated from the surface sterilised roots of several rice cultivars. Azospirillum strains from different rice cultivars varied in their nitrogen-fixing ability in vitro (Table 12) as with $^{15}N_2$ incorporation. Interestingly, the strains from a single cultivar possessed a certain degree of uniformity with respect to the nitrogen-fixing ability irrespective of the growth stage of the plant. Thus, Azospirillum cultures with high nitrogen-fixing activity, from rice cultivars Kalinga I & II Mahsuri, Pankaj, Padma and Vani exhibited uniformly high nitrogen-fixing activity. A gradual decrease in the nitrogen-fixing ability with respect to plant age was evident in Azospirillum cultures at different stages of the plant growth. Azospirillum isolated from the roots of cultivars receiving low levels of combined nitrogen, in general, exhibited greater nitrogen-fixing activity than the strains from the roots of unfertilised plants (Table 13). Results suggest that the nitrogen-fixing ability of cultures inhabiting in and around the roots can be enhanced following certain amendments, at least, in some rice cultivars.

Large variations in the nitrogen-fixing activity of the rhizosphere samples from 20 rice cultivars grown under uniform field conditions were noticed.

The rhizosphere samples were collected from the field grown plants and incubated in an atmosphere of labelled $^{15}N_2$. All other conditions were uniform excepting the variety from which the rhizosphere soil was collected. Striking differences in nitrogen-fixing potentials of rhizosphere soil from different varieties indicated the role of plant genotype.

Nitrogen fixation was stimulated in the rice rhizosphere following the rice straw incorporation under submerged conditions (Table 14). $^{15}N_2$ incorporation was low in unplanted soil compared to that of the rhizosphere soil. Addition of rice straw at higher levels (6 tons/ha) further enhanced nitrogen fixation in the rhizosphere soil. Obviously, supplementation with organic matter to the submerged soil stimulates the microbial proliferation and maintains the flooded soil under more reduced conditions favouring the nitrogen fixation. Nitrogen fixation was suppressed in the rhizosphere samples from ammonium sulphate treatments at 40 kg N/ha, with a further reduction at 80 kg N/ha. Interestingly, application of higher levels of rice straw (6 tons/ha) alleviated this suppression of ammonium sulphate. These results convincingly demonstrate the role of rice rhizosphere in maintaining the fertility status of the paddy soils and provides further information on the influence of rice variety, organic matter and combined nitrogen.

Considerable number of Azospirillum was noticed in freshly collected soil samples from submerged rice fields (Table 15). Striking stimulation of Azospirillum population occurred in rice straw amended and benlate amended field soils. However, the stimulation was at a lower degree when benlate and rice straw were applied together. It has been established that application of benlate keeps the submerged soil at a relatively high potentials for prolonged periods.

A survey was made on the occurrence of Azospirillum sp. in several weeds associated with rice and aquatic ecosystem. Nitrogen fixation was higher in cultures isolated from roots. The phyllosphere strains were less efficient in fixing nitrogen except for some plant species.

The influence of two organophosphate insecticides and natural products having insecticidal effect on nitrogen fixation was investigated in a field experiment (Table 16). The peak rhizosphere nitrogen activity was noticed after 60 days of transplanting which decreased further. All the compounds tested decreased the rhizosphere nitrogenase activity, although the extent of inhibition varied with individual compound. Neem products and organophosphate insecticides were less inhibitory on the rhizosphere nitrogenase activity, while the inhibition was more pronounced in pyrethrum and allitin treated samples.

Interestingly, nitrogenase activity was high in Azospirillum cultures isolated from the roots treated with quinalphos and phosalone as compared to the cultures from other treatments. This indicates that the influence of a particular compound varied with the site of nitrogenase activity. This finding further suggests the necessity for a careful evaluation of the effects of pesticides as nitrogen fixation in view of the differential nitrogenase activity in the rhizosphere and root inhabitants.

Our results show the importance of judicious management of mineral and organic fertilizers, selection of a suitable rice variety and other cultural practices would substantially alter the nitrogen contributions through biological nitrogen fixation. This would further emphasise the role of associative and free-living nitrogen fixers to the overall nitrogen economy of tropical paddy soils.

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Table 1. Some characteristics of the soils

Soil type	pH (1 : 1.25)	Organic matter	Electrical conductivity (mmhos/cm)	Total N (%)	CEC (me/100g)
Alluvial	6.2	1.6	0.6	0.09	18.6
Laterite	5.0	3.25	0.2	0.09	42.8
Acid sulphate saline	4.2	8.21	8.5	0.24	19.2
Acid saline	5.0	5.76	6.3	0.24	8.0

Table 2. Indigenous (Atmospheric) nitrogen fixation in Indian rice soils under two water regimes

Soil type	Atom % excess ^{15}N at the end of the incubation			
	Flooded condition	(SD ±)	Nonflooded condition	(SD ±)
Alluvial	0.123	0.021	0.053	0.003
Laterite	0.180	0.012	0.032	0.010
Acid sulfate (pokkali)	0.065	0.015	0.057	0.002
Acid saline (kaapadam)	0.031	0.006	0.015	0.006

Table 3. Influence of carbon sources on asymbiotic N_2 fixation in alluvial, laterite and acid sulphate (pokkali) soils under two water regimes

Carbon source	Flooded soil						Nonflooded soil					
	Alluvial		Laterite		Acid sulphate		Alluvial		Laterite		Acid sulphate	
	a	b	a	b	a	b	a	b	a	b	a	b
Unamended	0	0	0	0	0.008	0.6	0	0	0	0	0.075	5.6
Cellulose	0.756	25.6	0.792	38.0	0.073	5.5	0.819	27.0	0.299	7.2	0.036	2.0
Glucose	0.111	3.7	0	0	0	0	0.448	15.0	0	0	0	0
Sucrose	0.244	1.6	0.015	0.7	0	0	0.710	24.0	0.026	1.0	0	0
Malate	0.030	1.0	0	0	0	0	0	0	0	0	0	0
Rice straw	0.374	13.6	0.021	1.0	0.208	16.0	0.204	7.4	0	0	0.241	18.0

Differences are significant at 1% level. a = Atom % excess ^{15}N in the soil at the end of the 28-day incubation. b = Cumulative N_2 fixation (Kg N/ha). The soils were amended with 0.5% carbon source. Malate was added at 0.1 mM.

Table 4. Nitrogen fixation in an organic amended alluvial soil as influenced by moisture content and combined nitrogen

N-ppm	*Atom % excess ^{15}N at the end of incubation	(SD ±)	Nitrogen fixed (mg/kg)	Cumulative nitrogen fixation (kg/ha)
		Flooded condition		
0	0.753	0.045	12.8	25.7
20	0.648	0.017	11.0	22.0
40	0.491	0.132	8.3	16.7
60	0.423	0.069	7.2	14.4
80	0.398	0.138	6.8	13.5
100	0.383	0.083	6.5	13.0
		Nonflooded condition		
0	0.819	0.149	13.9	27.8
20	0.880	0.051	13.6	27.2
40	0.655	0.056	11.0	22.0
60	0.502	0.066	8.5	17.0
80	0.110	0.027	2.0	4.0
100	0.058	0.022	1.0	2.0

* CD between fertilizer levels at P 1% and 5% are 0.17 and 0.12 respectively.

Table 5. Influence of combined nitrogen and moisture regime on nitrogen fixation in a laterite soil amended with cellulose

N-ppm	*Atom % excess ^{15}N at the end of incubation	(SD ±)	Nitrogen fixed (mg/kg)	Cumulative nitrogen fixation (kg/ha)
		Flooded condition		
0	0.792	0.040	19.0	38.0
20	0.398	0.008	9.6	19.2
40	0.070	0.010	1.7	3.4
60-100	0	0	0	0
		Nonflooded condition		
0	0.299	0.015	7.2	14.5
20-100	0	0	0	0

*CD between fertilizer levels at P 1% and 5% are 0.17 and 0.12 respectively.

Table 6. Effect of combined nitrogen and moisture regime on nitrogen fixation in two acid (pokkali and Karapadam) soils amended with cellulose

Flooded condition					Nonflooded condition				
N-ppm	Atom % excess ^{15}N at the end of incubation	(SD ±)	Nitrogen fixed (mg/kg)	Cumulative nitrogen fixation (kg/ha)	N-ppm	Atom % excess ^{15}N at the end of incubation	(SD ±)	Nitrogen fixed (mg/kg)	Cumulative nitrogen fixation (kg/ha)
				Pokkali					
0	0.073	0.007	2.8	5.5	0	0.036	0.019	1.3	2.7
20-100	0	0	0	0	20-100	0	0	0	0
				Karapadam					
0	0.317	0.027	9.0	18.0	0	0.122	0.015	3.2	6.5
20-100	0	0	0	0	20-100	0	0	0	0

Table 7. Influence of alternate flooded and nonflooded (drained) conditions on acetylene reduction in an alluvial soil amended with cellulose

Treatment	Nitrogenase activity (n moles C_2H_2 formed/g/day)							
	Soil incubation (days)							
	2	4	8	13	16	20	30	48
NF (48d)	0.10*	0.15*	0.15*	0.08*	0.04*	0.02*	0.02*	0.06*
F (48 d)	0.18*	0.53+	38+	40+	70+	45+	11+	1.97+
NF (15 d)+F (33 d)	0.05*	0.08*	0.15*	0.02*	1.43+	4.52+	4.97+	8.60+
F (15 d)+NF (33 d)	0.20+	2.50+	40+	37+	24*	3.50*	2.47*	1.45*
F (7 d)+NF (7 d)+F (34 d)	0.18+	3.32+	36*	21*	43+	65+	0.27+	0.75+
NF (7 d)+F (7 d)+NF (34 d)	0.07*	0.01*	0.14+	4.10+	19*	4.50*	3.71*	0.02*
LSD								
P = 0.01	0.08	1.08	11.08	7.56	13.78	13.10	1.88	0.96
P = 0.05	0.06	0.76	7.90	5.26	9.83	9.34	1.34	0.68

F = flooded,
NF = nonflooded,
Figures in the parenthesis indicate the number of days under the respective treatment
* nonflooded and + flooded conditions at the time of the assay.

Table 8. Influence of alternate flooded and nonflooded (drained) conditions on acetylene reduction in a Pokkali soil amended with cellulose.

Treatment	Nitrogenase activity (n moles C_2H_4 formed/g/day) Soil incubation (days)							
	2	4	8	13	16	20	30	48
NF (48 d)	0.17*	0.20*	0.22*	0.22*	0.14*	0.13*	0.07*	0.04*
F (48 d)	0.27+	3.11+	76+	68+	140+	86+	87+	42+
NF (15 d)+F (33 d)	0.15*	0.16*	0.15*	0.05*	2.85+	7.0+	7.63+	4.24+
F (15 d) + NF (33 d)	0.27+	7.74+	82+	74+	98*	1.97*	1.57*	1.10*
F (7 d) + NF (7 d)+F (34 d)	0.27+	9.51+	19*	7.30*	88+	156+	19.50+	11+
NF (7 d) + F(7 d)+NF (34 d)	0.16*	0.20*	0.22+	8.76+	8.80*	8.10*	4.30*	2.96
LSD								
P = 0.01	0.08	2.99	15.01	11.28	19.77	7.58	11.63	14.17
P = 0.05	0.06	2.13	10.71	8.05	14.11	5.41	8.29	10.11

F = flooded,
NF = nonflooded,

Figures in parentheses indicate the number of days under the respective treatment

* nonflooded and + flooded conditions at the time of the assay.

Table 9. Effect of pesticides on nitrogen fixation in submerged paddy soils.

Treatment	Atom % excess ^{15}N at the end of the incubation ± standard deviation *				
	Alluvial	Laterite	Acid sulphate saline		Acid saline
			Pokkali	Kari	Karapadam
No pesticide	0.277 ± 0.168 (4.7)	0.237 ± 0.077 (5.7)	0.005 (0.2)	0.012 ± 0.002 (1.5)	0.055 ± 0.010 (1.6)
+ Carbofuran	0.786 ± 0.067 (13.4)	0.296 ± 0.063 (7.2)	0 (0)	0 (0)	0.067 ± 0.006 (2.0)
+ Benomyl	0.542 ± 0.113 (9.2)	0.427 ± 0.044 (10.3)	0.017 ± 0.001 (0.6)	0.046 ± .006 (5.6)	0.028 ± 0.004 (0.8)
+ Gamma-BHC	0.754 ± 0.071 (12.8)	0.161 ± 0.022 (4.0)	0.033 ± 0.005(1.2)	0.013 ± 0.003 (1.6)	0.046 ± 0.005 (1.3)

* LSD between treatments at PI and 5% is 0.063 and 0.050 respectively.

Figures in parentheses indicate N_2 fixed mg.kg^{-1} soil.

Table 10. N_2-fixing populations in pesticide-amended submerged soils

Soil	*Azospirillum* sp.			*Azotobacter*			Anaerobic N_2 fixers		
	No pesticide	Carbofuran	Benomyl	No pesticide	Carbofuran	Benomyl	No pesticide	Carbofuran	Benomyl
	(10^6 g^{-1} dry soil)			(10^3 g^{-1} dry soil)			(10^6 g^{-1} dry soil)		
Alluvial	5.4	26	34	1.3	1.5	1.3	4.8	18.4	34
Laterite	48	0.48	32.0	0.4	1.7	0.2	2.2	22	0.26
Karapadam (acid saline)	3.2	1.6	9.0	1.0	0.8	0.6	18.4	5.6	7.0

Table 11. $^{15}N_2$ incorporation by *Azospirillum* sp. from different rice cultivars.

Rice cultivar from which *Azospirillum* was obtained	Nitrogen fixed mg/30 ml medium	Atom % excess ^{15}N at the end of the incubation
Kalinga I	15.70	11.84
IR.8	13.00	9.77
MR.1550	6.50	4.89
Pankaj	4.80	3.62
Jayanthi	2.80	2.10
Pusa 2-21	2.40	1.83
Padma	0.30	0.21

Table 12. Nitrogen-fixing ability of *Azospirillum* isolated from roots of rice cultivars grown without combined nitrogen

Rice cultivar (without fertilizer nitrogen)	Nitrogen fixed $mg.g^{-1}$ malate added by *Azospirillum* cultures. Mean of 3 replications		
	Age of the plant (days)		
	15	30	45
Kalinga I	7.42	8.72	5.15
Kalinga II	7.28	6.65	4.70
Shakti	6.47	7.39	4.93
CR.1014	6.13	6.13	6.58
Mahsuri	5.37	5.71	4.77
Pankaj	5.11	5.88	4.67
Pusa 2-21	4.31	4.31	4.80
CR.1009	4.26	4.26	3.75
Padma	4.55	5.11	2.56
Supriya	4.97	7.10	6.70
Vani	4.41	3.10	3.10
Jaya	3.61	2.38	2.80
Jagannath	3.92	3.92	3.92
NC.1281	1.16	1.15	1.55
T.141	2.21	3.71	1.47
MR.1550	1.68	2.24	1.05
Jayanthi	0.77	1.80	2.17

Table 13. Nitrogen-fixing ability of *Azospirillum* isolates from roots of rice cultivars grown with fertilizer nitrogen

Rice cultivar fertilized (60 kg N/ha)	Nitrogen fixed mg.g^{-1} malate added by *Azospirillum* cultures. Mean of 3 replications		
	Age of the plant (days)		
	15	30	45
Kalinga I	8.15	8.75	3.98
Kalinga II	9.27	9.35	5.25
Shakti	7.28	7.42	5.10
CR.1014	7.70	8.91	7.35
Mahsuri	8.54	9.10	9.24
Pankaj	6.70	7.63	7.77
Pusa 2-21	6.10	7.45	3.85
CR.1009	5.51	5.81	3.43
Padma	4.31	5.71	4.55
Supriya	6.58	8.05	6.93
Vani	6.79	5.10	3.43
Jaya	3.92	4.76	3.61
Jagannath	4.24	4.34	3.75
HC.1281	2.80	2.28	2.35
T.141	3.00	3.78	2.24
MR.1550	1.75	2.45	1.26
Jayanthi	2.17	2.32	2.66

Table 14. Nitrogen fixation in rhizosphere samples from rice straw and ammonium sulphate amended soil

Treatment	Atom % excess ^{15}N at the end of the incubation	± SD	mg N_2 fixed/ kg soil
Non-rhizosphere soil	0.30	0.12	5.2
Rhizosphere soil	1.30	0.10	22.2
+ Rice straw (3 t/ha)	1.50	0.10	25.5
+ Rice straw (6 t/ha)	1.70	0.06	29.4
# 40 kg N/ha	1.17	0.15	19.9
+ 80 kg N/ha	0.97	0.06	16.5
+ Rice straw (3 t/ha) + 40 kg N/ha	0.93	0.23	15.9
+ Rice straw (3 t/ha) + 80 kg N/ha	0.77	0.15	12.9
+ Rice straw (6 t/ha) + 40 kg N/ha	1.53	0.06	25.8
+ Rice straw (6 t/ha) + 80 kg N/ha	1.07	0.15	18.2
LSD 5%	0.21		
1%	0.29		

Table 15. Population of *Azospirillum* as influenced by rice straw and benomyl in rice fields

Treatments	* Population of *Azospirillum* (m.p.n x 10^6/g soil)
Unamended	0.26
+ Rice straw (3 t/ha)	33
+ Benlate (3 kg a.i./ha)	21
+ Rice straw + Benlate	13

* Average of five replicates.

Table 16. Effect of natural and synthetic insecticides on rhizosphere nitrogenase and *Azospirillum* isolates from roots

Treatments	Nitrogenase activity of rhizosphere soil *n moles of C_2H_2 formed/g fresh soil/day			Nitrogenase activity of *Azospirillum* isolated from roots (*n moles of C_2H_2 formed/ml medium/day
	60 DAT**	75 DAT**	85 DAT**	
Control	101 ± 6.0	34 ± 3	3.12 ± 0.15	14.88 ± 0.55
Neem extract	58 ± 2.5	31 ± 2	1.92 ± 0.40	15.36 ± 0.11
Neem oil	43 ± 1.0	3.36 ± 0.35	1.92 ± 0.52	16.32 ± 0.75
Pyrethrum	20 ± 1.5	6.80 ± 0.35	2.16 ± 0.45	17.76 ± 0.23
Allitin	32 ± 1.6	4.10 ± 0.20	1.92 ± 0.75	17.10 ± 0.35
Quinalphos	40 ± 3.3	3.36 ± 0.34	1.92 ± 0.52	23.28 ± 0.80
Phosalone	41 ± 3.2	4.10 ± 0.32	2.79 ± 0.25	18.48 ± 0.15

* Nitrogenase activity ± standard deviation of the mean of triplicate samples.

** DAT = days after transplanting. The rhizosphere soil samples were collected 10 days after the application of respective compounds. The nitrogenase activity of the *Azospirillum* cultures isolated from the roots (93-day plants) under different treatments was assayed after incubation in C_2H_2 for 24 h.