Azospirillum ASSOCIATED WITH RICE IN ACIDIC SOILS OF COASTAL KARNATAKA AND ITS POSSIBLE USE AS A BIOINOCULANT

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C E R T I F I C A T E

This is to certify that the thesis entitled "Azospirillum ASSOCIATED WITH RICE IN ACIDIC SOILS OF COASTAL KARNATAKA AND ITS POSSIBILE USE AS A BIOINOCULANT" submitted by Mr. M. GOVINDAN, for the degree to DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY of the University of Agricultural Sciences, Bangalore, is a record of bonafide research work done by him during the period of his study in the University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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INTRODUCTION

I. INTRODUCTION

The advancing crop production technology, development of high fertilizer responsive crop varieties and pressure for intensive farming demand huge inputs, of which nitrogenous fertilizers take a major share. The ever increasing and exploding population, together with the hiking price of petroleum and petroleum based products tightens the global situation to such a rigorous extent that the nitrogenous fertilizers have not only become costlier but also quite insufficient to meet the demands of the farming community as a whole. Convincing evidences have pointed out that biological nitrogen fixation (BNF) requiring a relatively simple and low cost technology, easy to execute and largely of renewable sources of energy has a tremendous role to play in the immediate future of agriculture, especially in the developing and underdeveloped countries.

Global gains of nitrogen have been estimated to be 175 million tons $year^{-1}$ through biological fixation alone and 35 million tons $year^{-1}$ through precipitation (Burns and Hardy, 1975). The acquisition of nitrogen from the atmosphere is accomplished by unique group of organisms which fix nitrogen because they possess the enzyme 'nitrogenase'. World's most staple food crops (maize, wheat, rice, sorghum, millet) and grasses have long been thought to

have no potential for biological N_2 fixation. This concept was thrashed down by the startling discoveries of newer and hitherto unexplained and untrapped diazotrophs and nonsymbiotic nitrogen fixation was unequivocally demonstrated in rice, maize, pearl millet and a variety of tropical grasses (Yoshida and Ancajas, 1973; Dobereiner and Day, 1976) . Enlisted in this category is Azospirillum sp. which during the past 20 years found an ever increasing scientific interest. These soil bacteria fix atmospheric nitrogen, live in close vicinity to the roots of grain crops and forage grasses and have a wide geographic distribution (Neyra and Dobereiner, 1977; Michielis et al 1989). The beneficial effects on plants by Azospirillum inoculation are well documented (Michielis et al., 1989; Bashan and Levanony, 1990)

Rice is the staple food of approximately one half of the worlds population and is cultivated in an area of 143 million hectares. By 2000 AD, India shall have to produce 225-240 million tonnes of food grains to nourish the projected population of about a billion. Achieving this goal by increasing crop production needs huge input of fertilizer nitrogen. Hence developing a suitable biofertilizer for rice needs priority. Azospirillum inoculation favoured rice yield under favourable conditions (Subba Rao et al., 1979; Watanabe and Lin, 1984; Rao et al., 1987). Varying

agroclimatic and soil conditions affect the field performance of the organism and makes it extremely difficult to put forth a blanket recommendation to adopt the inoculation technology. The major environmental factors governing the establishment and performance of the organism in soil are the seasonal variations of temperature, soil moisture, nutrients and soil pH. Further, the acidic soils with a pH ranging from 4.5 to 6.5 cause iron and aluminium toxicity. The soils of coastal Karnataka are having low pH, high aluminium and iron. Hitherto no attempt has been made to evaluate the native population of the diazotroph, Azospirillum in these soils and study its biocoenosis with rice to exploit its potentiality as a microbial inoculant. Hence the present study has been formulated with the following objectives.

1. To study the occurrence of Azospirillum in acidic soils of coastal Karnataka.
2. To find out the population of native Azospirillum in different seasons and at different depths of soil.
3. To study the effects of soil amendments on the native population of Azospirillum
4. To isolate Azospirillum and to screen, under both in vivo and in vitro conditions, for an efficient isolate that can be used as a biofertilizer for rice grown in acidic soils of coastal Karnataka.

5. To study the inoculation effect of selected Azospirillum on a local variety of rice at graded levels of fertilizer nitrogen.
6. To find out the survival of selected Azospirillum in different carrier materials.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The fundamental importance of nitrogen for crop growth was established as early as 1838 by the classical experiments of Boussingault. The advancing crop production technology, development of high fertilizer responsive crop varieties and pressure for intensive farming demand huge inputs, of which nitrogenous fertilizers take a major share. Convincing evidences have pointed out that Biological Nitrogen Fixation (BNF) requiring a relatively simple and low cost technology, easy to execute and largely of renewable sources of energy has a tremendous role to play in the immediate future of agriculture.

Some of the most promising organisms capable of colonizing roots in large numbers and exerting beneficial effects on plants belong to the genus Azospirillum. Results from field experiments carried out since 1974 have shown that Azospirillum inoculation does promote yield under certain environmental and soil conditions.

2.1 ASSOCIATIVE NITROGEN FIXATION IN RICE

About 75% of the 143 million hectares of rice lands are low lands (wet lands). Evidence that flooding favour N_2 fixation in soils have been obtained from acetylene reduction assays (Yoshida and Ancajas, 1971), long term field trials (Watanabe et al., 1981), and nitrogen balance

studies (Ventura and Watanabe, 1983). Rice soils offer unique conditions for the ability of aerobic, microaerophilic and anaerobic N_2- fixing microorganisms (Yoshida and Ancajas, 1973; Watanabe, 1978; Rao 1980). Burns and Hardy (1975) estimated the BNF in rice fields as about 30 Kg N ha^{-1} yr^{-1}. Total N fixed by low land rice fields was calculated as 3.2 million t N yr^{-1} and the theoretical estimate on the potential of BNF due to associative N_2 fixers was estimated to be 40 Kg N ha^{-1} (Watanabe and Roger 1984). Studies using ^{15}N technique confirmed that bacteria in association with rice roots and submerged portions of shoots can fix N_2 and provide at least a part of this fixed N_2 to the rice plant (Eskew et al., 1981, Ito et al., 1980, Yoshida and Yoneyama, 1980). 15 to 30% of nitrogen fixed by free living diazotrophs was assimilated by rice and about 50% of it went into the composition of soil humus (Kalininskaya et al., 1981).

Different genera of N_2 fixing bacteria isolated from wet land rice include Azospirillum (Lakshmikumari et al 1976), Pseudomonas (Watanabe and Barraquio, 1979), Enterobacter and Klebsiella (Watanabe et al., 1979).

2.2 RECOGNITION OF Azospirillum

The bacterium first described in 1922 by Beijerinck as Azotobacter spirillum was later renamed as Spirillum lipoferum. (Beijerinck, 1925). This bacterium was able to fix

atmospheric nitrogen in enrichment culture. Dobereiner and Day (1976) reported the association between Digitaria decumbens and S. lipoferum. In this host bacteria are in close proximity to the vascular tissue of the plant and they have easy access to the photosynthetic products and in turn fixed nitrogen which is supplied directly to the plant. They also reported that S. lipoferum is a very common root and soil inhabitant in the tropics. Subsequent taxonomic studies led to the creation of a new genus, Azospirillum. This genus defined by Tarrand et al. (1978), comprised two species: A. brasilense and A. lipoferum. Another species, A. amazonense was isolated from sugarcane (Saccharum officinarum) and sugar sorghum roots (Magalhaes et al., 1983). It is more acid tolerant and has also been found in roots of palm trees in the Amazon region. Reinhold et al. (1987a) isolated and described A. halopraeferens from roots of kallar grasses grown on salt affected soils in Pakistan. This species showed some adaptation to salinity and showed remarkable growth and N_2 fixation at $40^{o}C$ with 0.25% salt in the growth medium. Herbaspirillum seropedicae was isolated from the roots of maize, rice and sorghum in the Brazilian savanna soils. Its N_2 fixation is tolerant to lower O_2 and lower pH than that of Azospirillum (Dobereiner et al., 1988). Khammas et al. (1989) discovered a new species, A. irakense.

2.3 BIOGEOGRAPHY OF *Azospirillum*

Since 1974, when *Azospirillum* rhizocoenosis was first reported, a large volume of information has been accumulated. Dobereiner *et al.*, (1976) suggested that the N_2 fixing *Spirillum* is a very common root and soil inhabitant of the tropic. Subsequent studies revealed that these bacteria are very common in many parts of the world tropical, temperate or subtropical and in association with different plant species like grasses, rice, maize, wheat, barley, rice, oats (Dobereiner and De Polli, 1980), and plantation crops (Govindan and Purushothaman, 1985).

S. lipoferum was isolated from surface sterilized rice roots (Lakshmikumari *et al.* 1976). Rao *et al.*, (1978) reported the ubiquitous presence of nitrogen fixing *Spirillum* in different soil types (Alluvial, Laterite, Pokkali and Kari) under both upland and low land conditions. Host plant specificity in the infection of *Azospirillum* has been demonstrated. Ninety five per cent of the isolates from rice were *A. brasilense* nir^- (Baldani and Dobereiner, 1980). However many reports contradicting the above have appeared. Ladha *et al.* (1987) reported that most of the isolates from surface sterilized rice roots were *A. lipoferum*. *Azospirillum* constituted about 1% of the total aerobic heterotrophs of endorhizosphere. Dung *et al.*, (1988) reported *Azospirillum* to be frequent colonizer in the

rhizosphere of rice grown in Vietnam. Balasubramanian and Prabhu (1989) reported the occurrence of A. halopraeferens associated with rice roots. Recently khammas et al. (1989) isolated Azospirillum irakense from the rice roots and rhizosphere soil.

2.4 VARIABILITY OF POPULATION OF Azospirillum IN THE RHIZOSPHERE

A population is characterized by its density, size and composition. The composition of N_2 fixing populations exhibit a large degree of variability. This variability can be seen in the occurrence, or relative abundance of genera, species and strains.

Smith et al. (1982) made a survey of different counties of Florida, looking for highly active rhizospheres. They could demonstrate a very patchy pattern of distribution of active sites and occurrence of several genera. For Azospirillum the distribution seems very erratic. (Reynders and Vlassak, 1978).

Besides occurrence dominance also varies greatly among comparable ecological niches. Rennie (1980) studied three Agropyron species. In one, the dominant N_2 fixing bacteria comprised of Azospirillum and Azotobacter while in the other two it was Erwinia herbicola.

At the species level, dominance can vary. In Azospirillum, the contention has been made (Baldani and Dobereiner, 1980) that A. lipoferum is dominant in the rhizosphere of grasses with a C_4 photosynthetic pathway whereas A. brasilense is dominant in C_3 plants. But this is certainly not an absolute rule as the converse situation has often been met (Heulin et al., 1982; Ladha et al., 1982).

Within a single species of Azospirillum, differences have been found between strains isolated from comparable habitats or even from the same habitat (Balandreau, 1986). Dobereiner and Baldani (1979) found some diversity among Azospirillum strains concerning their resistance to streptomycin, tetracycline, gentamycin, erythromycin and chloramphenicol.

Nur et al., (1980) found very large differences in growth and acetylene reduction rates with various carbon sources. Rinaudo et al., (1986) compared 251 morphological, physiological and biochemical features in 50 Azospirillum strains, mostly isolated from rice in Senegal. Their numerical taxonomy study indicated different "phenons" in Azospirillum. These do not fit into the classical separation into two or three species.

2.5 ESTABLISHMENT OF THE Azospirillum - PLANT ROOT ASSOCIATION

The so-called rhizocoenosis formed between Azospirillum and plant roots depend on an intimate mutual interaction between both partners. This interaction takes place in the rhizosphere or within the root tissue, but no specialized structures comparable to the legume root nodules induced by Rhizobium are formed. The association can therefore best be described as a mere colonization of the rhizosphere, rhizoplane and/or the root interior. (Michiels et al., 1989). This colonization is the result of a selective environment of the microorganism best adapted to the ecological niche formed by the root environment.

Azospirilla dispose of several properties that are thought to promote competitiveness in the rhizosphere. They include chemotaxis, attachment to roots, colonization of the root interior, production of cysts, utilization of a wide array of carbon and energy sources, production of growth substances etc.

2.5.1 Chemotaxis

Azospirillum is chemotactically attracted to the rhizosphere in response to a variety of amino acids, mono and disaccharides and organic acids (Barak et al., 1983; Reinhold et al., 1985; Heinrich and Hess, 1985). Chemotaxis was shown to be strain specific and attuned to the

composition of host root exudate. Azospirillum isolated from the C_4 plants kallar grass and maize, showed strong attraction to malate but not to oxalate. Malate is the dominating organic acid exuded by kallar grass and presumably also by maize, while oxalate is hardly detectable. Azospirillum strains isolated from wheat responded strongly to oxalate but not to malate. Oxalate is the major organic acid in the root exudate of wheat (C_3 plant) while malate is released in much smaller quantities (Reinhold et al., 1985).

Azospirilla are microaerophilic bacteria, attracted to self created O_2 gradients (Barak et al., 1982). This property may aid root colonization since active plant roots of rice will have microaerophilic condition even under submerged conditions due to transportation of O_2 to the root zone.

Azospirillum migrates towards growing wheat root after a lag of 24 hr and the main factors affecting motility are soil moisture, soil type and duration of plant growth prior to bacterial application (Bashan, 1986).

2.5.2 Attachment

Success of inoculation depends on the efficient colonization of the bacteria in the root environment. A. brasilense sp.7 was adsorbed to the seedlings of pearl

millet in a matter of seconds to root hairs and to old epidermal cells. (Umali-Garcia et al., 1980). The number of cells adsorbed was more than other gram negative bacteria studied. The attachment is in a polar fashion to root hairs, epidermal cells and mucigel but may also occur in clumps (Patriquin et al., 1983). Adherence of A. brasilense Cd to corn roots increased during the first 90 min and attained a maximum level within 4.5 hr of incubation (Okon and Kapulnik, 1986). Maximum binding of strain Cd occurred on the corn roots at pH 6.1. It is possible that at this pH the charges on the corn roots and on strain cd are such that minimal repulsion occur. Adsorption of azospirilla is affected by bacterial strain, host plant and part of the root involved (Umali-Garcia et al., 1980, Jain and Patriquin 1984, Bashan and Levanony, 1987b).

Usually, azospirilla do not attach themselves to the root system in a uniform pattern. On inoculation A. brasilense sp.7 was found to have preferential attachment to the root elongation zone in wheat (Kapulnik et al., 1985) and to the root hairs in pearl millet (Umali-Garcia et al., 1980).

Murty and Ladha (1987) found differences in the pattern of attachment of a particular strain of Azospirillum (A. lipoferum strain 34 H) between two varieties of rice (IR

42 and IR 50). The bacterium did not colonize the root tips of IR 42, but colonized this region in IR 50 within 24 hr after inoculation. In the early stages most of the bacteria were embedded in the ruptured mucigel below the root cap cell of IR 42. Mucigel was hardly detectable in IR 50. While the root hair primordia of IR 50 were heavily colonized by this bacterium within 24 hr the root hairs of IR 42 were colonized 48 and 72 hr after inoculation.

Bashan et al. (1986) suggested that adsorption of A. brasilense Cd on wheat roots can be of a weak active process.

Tabary et al. (1984) purified a lectin from rice embryos, labelled with ^{14}C acetic anhydride and showed it to interact and bind Azospirillum isolated from the rhizosphere of rice. No interaction between bacteria isolated from the rhizosphere of maize or E. coli and rice lectin was observed.

Sukiman and New (1990) studied the relationship between colonization and initial adsorption of Azospirillum to rice roots. Azospirilla adsorbed on the roots of six day old seedlings were counted by microscopy of the three sites on the roots and by available count of the terminal 2 cm of the roots. On rice, Sp. Br 14 gave highest number of adsorbed bacteria on the whole root and on the root cap and root hairs. Strain cd adsorbed significantly better than Sp.Br on

the root epidermis and was equally numerous on the other parts of the root, but was significantly under-represented in the viable count of the whole root. Strain Sp.7 adsorbed worse of all strains at all three sites, but this was not reflected in the viable count.

2.5.3 Internal Root Colonization

Azospirillum strains can penetrate the roots of their hosts and establish in high numbers in the intercellular spaces between the epidermis and cortex or in the outer cortical layers (Okon et al.,1977, Patriquin and Dobereiner, 1978, Umali-Garcia et al., 1978)·With inner root invasion, the ecto and endorhizosphere are usually colonized by different strains of Azospirillum (Baldani et al., 1986, 1987). Azospirillum was found to colonize the mucigel of rice roots within 30 minutes of inoculation (Lakshmi et al., 1977). The bacteria was observed in the inner regions of root hairs, epidermal and cortical cells of rice roots. Azospirillum can enter the roots by active penetration or through pre-existing lesions generated by emerging branches on the main roots or by contact of the growing root with soil particles (Michiels et al., *1989*). *No* viable intracellular associations have been reported so far.

A. brasilense Cd when inoculated on to wheat roots, multiplied and formed aggregates on the root surfaces and

established as internal root population. Washing the roots failed to remove the internal population (Bashan et al., 1986).

2.5.4 Bacteriocinogenic activity of Azospirillum

Many strains of A. brasilense and A. lipoferum produce bacteriocin (Olivira and Drozdowicz, 1981). However their role in the root environment is not clearly understood. Olivira and Drozdowicz (1988) showed that introduced bacteriocin persisted in autoclaved soil. The bacteriocin activity was present on the surface of extracted wheat roots and the bacteriocinogenicity of the producer strain persisted even during its proliferation in soil. A carbophenem antibiotic TAN-458 was isolated from the culture filtrate of Azospirillum (Kintaka et al., 1985).

2.5.5 Cyst formation

Vegetative cells of Azospirillum have the capacity to form non-motile, highly refractive, encapsulated C forms containing abundant poly β- hydroxy butyrate granules (Eskew et al., 1977; Sadasivan and Neyra, 1987). These forms which show increased resistance to desiccation and ultra violet and γ - irradiation are considered as cysts and may help to survive under unfavourable conditions (Lamm and Neyra, 1981).

2.5.6 Fe-acquisition system

Several Azospirillum strains possess very efficient Fe-acquisition system. (Bachawat and Ghosh, 1987). Under iron deficient conditions A. brasilense was shown to secrete a catechol type of siderophore (Spirilobactin) into the growth medium. A high affinity iron transport system capable of ^{59}Fe uptake from a $^{59}Fe^{3}$ -spirilobactin complex was found to be induced in this bacterium grown under iron deficiency.

2.5.7 Metabolic diversity

Azospirilla have a well adapted and highly versatile metabolism that allows them in a C-rich, N-poor rhizosphere, rhizoplane and/or endorhizosphere environment. Azospirilla prefer organic acids like malate, succinate and lactate. There are marked interspecies differences in the pattern of carbohydrate use. A. lipoferum readily utlises glucose and A. amazonense sucrose, whereas A. brasilense and A. halopraeferens cannot grow on these substrates efficiently. Azospirillum spp. differ in the utilization of amino acids also (Hartmann, 1988).

Azospirillum can grow and fix N_2 using polymeric substrates such as straw and xylan (Halsall et al., 1985) and hemicellulose (Ladha et al., 1986). In co-culture with cellulose degrading bacteria Azospirillum can fix nitrogen (Halsall and Gibson, 1985). Tien et al. (1981) reported the production of a polygalacturonic acid transeliminase by

several strains; which may play a role in penetration of root tissues. Myers and Hubbell (1987) reported that A. brasilense Sp.7 was not able to make use of polygalacturonic acid or pectin as a C source. A. irakense produces pectate lyase and pectin methyl esterase and can fix nitrogen when pectin is the sole carbon source (Khammas and Kaiser, 1991). The four other species of Azospirillum failed to show pectinolytic activity.

Azospirilla, can store C and energy in the form of poly-β-hydroxybutyrate granules (Okon et al., 1976). PHB granules serve as an electron sink and accumulate when respiratory electron acceptors (O_2, NO_2^-, $NO3^-$, $N2O$) are limited (Zimmer et al.,1984, Tal and Okon, 1985). They can support growth and respiration under the microaerobic conditions that occur during N_2 fixation, increase resistance to dessication, to osmotic shock and pressure, and to ultra-violet radiation (Tal and Okon, 1985).

A. lipoferum strains can grow autotrophically using H_2 and CO_2. (Pedrosa et al., 1980). They possess an uptake hydrogenase that allows them to use H_2 as an energy source for metabolism and a ribulose 1,5-biphosphate carboxylase for fixation of CO_2 (Malik and Schlegel, 1981). Uptake hydrogenase activity was also reported in A. brasilense Sp.7 (Chan et al., 1980), and it is thought to increase the

efficiency of N_2 fixation in several ways. The predominant N_2 fixing bacteria isolated from histosphere (endorhizosphere) of rice roots, Pseudomonas sp. and A. lipoferum were H_2 oxidising chemolithotrophs (Gowda and Watanabe, 1983).

Species of Azospirillum differed in their chemolithotrophic ability. Among the 33 strains of Azospirillum sp. studied, all the A. brasilense were non chemolithotrophs and there were two groups among the A. lipoferum strains, of which some of them have chemolithotrophic ability (Gowda, 1984). A. lipoferum was found to have H_2 dependent N_2 fixing ability under chemolithotrophic condition, and H_2 supported N_2 fixation in association with rice seedlings under monoaxenic conditions (Gowda and Watanabe, 1985).

A. lipoferum has to be classified as a part of the copiotroph microflora. It might have its natural habitat at special exudation sites on the root surface or in the endorhizosphere where the substrate concentration is higher. Kloss et al. (1986) isolated an Azospirillum strain belonging to the oligotroph microflora, being adapted to the low substrate concentrations in the rhizosphere and capable of supplying plant available nitrogen.

Azospirilla can grow using NH_4^+. If it is not available in the medium, NH_4^+ can be produced either by fixation of

molecular N_2 or by assimilatory NO_3 reduction (Michiels et al., 1989). The latter process allows azospirilla to grow using NO_3, NO_2 or NO as the sole N source. Many strains of A. lipoferum and A. brasilense, but not A. amazonense dissimilate NO_3 and NO_2 under a severe O_2 limitation (Eskew et al., 1977; Magalhaes et al., 1983). Some strains are even able to bring about denitrification, where NO_2 can be further reduced to the gaseous compounds (Krieg and Dobereiner, 1984).

2.5.8 Osmotolerance

In the different species of Azospirillum tolerance to high concentrations of sodium chloride, sucrose or polyethylene glycol increased in the order A. amazonense, A. lipoferum, A. brasilense and A. halopraeferens (Hartmann et al., 1991). In A. irakense growth occurred in the presence of 1 to 3% NaCl (Khammas et al., 1989).

Nitrogenase activity was more sensitive towards salt stress than cellular growth on combined nitrogen (Rao and Venkateswarlu, 1985). Half maximal in vitro nitrogenase activity of A. amazonense was obtained at 110 mm NaCl, as was the case with whole cell nitrogen fixation (Hartmann, 1987). In osmotic stress conditions, growth of A. brasilense and A. halopraeferens was stimulated by glutamate or proline, whereas no effect was found with A. lipoferum or A. amazonense (Hartmann, 1987). The latter two species

efficiently used glutamate and proline as carbon and nitrogen source (Hartmann 1988). Therefore, A. lipoferum and A. amazonense may not be able to use amino acids as compatible solutes during osmotic stress. Betaine glycine stimulated nitrogen fixation in A. brasilense and A. halopraeferens at otherwise inhibitory osmotic stress. Even low concentrations of glycine betaine were sufficient to improve osmotolerance. A. lipoferum could not use them as osmoprotectants (Hartmann,1988). A. halopraeferens had a high affinity uptake for choline.

Osmotolerant strains scavenge potential osmoprotectants such as proline, betaine or choline, originating from the plant. The halophyte kallar grass where A. halopraeferens resides in the rhizoplane accumulated high levels of betaine and proline at high salinity (Sandhu et al., 1981). A. halopraeferens is characterised by its sodium requirement (Hartmann, 1987; Reinhold et al., 1987).

2.6 INTRINSIC ANTIBIOTIC RESISTANCE OF Azospirillum

Strain differentiation is a major problem and it could be done by morphological, serological or genetic traits. Ladha et al. (1982) used immunological tests to identify Azospirillum strains.

Azospirillum strains have different tolerance level to various antibiotics. Most Azospirillum strains were killed

at 5 ppm streptomycin concentration and use of 20 ppm streptomycin helped to isolate higher percentage of resistant strains (Baldani and Dobereiner, 1980). Franche and Elmerich (1981) found that all the wild type strains of A. lipoferum and A. brasilense were sensitive to streptomycin, rifampicin, nalidixic acid, cephaloridin and cefalotin. Singh and Wensel (1982) reported that all the strains of A. brasilense and A. lipoferum were resistant to trimethoprim except A. lipoferum A 23 which was highly sensitive. Magalhaes et al. (1983) observed that all the A. amazonense strains were resistant to penicillin, but relatively tolerant to chloramphenicol and erythromycin.

2.7 METHODS OF Azospirillum INOCULATION

Root inoculation with Azospirillum to rice seedlings was done by dipping the seedlings in trays filled with the aqueous slurry of peat based Azospirillum for a period of 4-8hr. (Rao et al., 1983)

Two methods of Azospirillum application viz (1) nursery and main field treatment and (2) main field treatment alone in the presence of graded levels of fertilizer N were evaluated in IR 50 rice (Kumar and Balasubramanian, 1989). Both the treatments markedly increased the number of tillers, plant dry weight, total N uptake, grain and straw yields over the respective fertilizer N controls. The number

of tillers per plant and mean grain yield with 50% N and Azospirillum application in the nursery and mainfield were on par with 100% N alone. However, the mean straw yield obtained at 75% N ha^{-1} with Azospirillum treatment in the nursery and main field was statistically on par with 100% N ha^{-1} alone. The results indicated that Azospirillum biofertilizer treatment in the nursery and mainfield was better and could help to save 25 to 50% fertilizer N without reducing the grain or straw yield.

2.8 CARRIERS IN INOCULANT PRODUCTION

Development of a suitable carrier material is required for successful field application of any biofertilizer. Iswaran et al. (1969) reported that rhizobial counts in Indian peat based inoculants were very high as compared to soil based inoculant. Moreover its survival was also better in peat. Kandaswamy and Prasad (1971) showed that growth of Azospirillum spp. was better at 28°C than at 35°C with lignite as the carrier. The highest count obtained in this carrier was 13.14×10^8 which was more than the highest count found in soil culture (1×10^8).

Iswaran and Apte(1972) observed that Indian peat was satisfactory as a carrier for Azotobacter. He noticed that counts increased slowly and remained more or less constant after 8 weeks. Sharma and Verma (1973) explored the possibilities of the use of lignite as carrier and found it

suitable for R. leguminosarum, R. trifolii and R. meliloti. Calcium carbonate was found to be a suitable neutralizing agent in inoculant preparation (Iswaran et al., 1969; Kandaswamy and Prasad, 1971; Iswaran and Apte,1972).

Moisture content of 45-55 per cent appeared most suitable to retain the viability of rhizobia for longer period of storage in lignite (Sharma and Verma, 1973). Jayaprakash (1980) studied the survival of R. phaseoli TAL.309 in three different carrier materials. Peat was found to be superior to lignite which was in turn superior to soil compost mixture. Tilak et al. (1979) tested several carrier materials including peat for use with Azospirillum. Soil and farm yard manure mixed 1:1 and sterilized for 3hr daily on three consecutive days was found to be a suitable carrier.

2.9. ESTABLISHMENT OF THE INOCULATED BACTERIA

It is essential to determine the establishment, multiplication and functioning of the introduced microorganisms under actual field conditions. Azospirillum inoculation to maize resulted in increased numbers of the introduced organism at early stages of plant growth which later declined (Hegazi et al., 1979). Jagnow (1981) reported that A. lipoferum could survive better in the root zone of plants and have a high degree of protection from freezing, thawing and drought.

Inoculated Azospirillum was found to decline rapidly from the rhizosphere of grasses indicating that the bacteria did not establish in the soil in high numbers. Bashan (1986) reported that when A. brasilense was inoculated to wheat root in normal soil, the population dropped by 90% or more within two weeks as a consequence of competition from other microbes. Establishment of the inoculated Azospirillum was traced in wet land rice using antibiotic resistant A. lipoferum (Nayak et al. 1986). Results indicated that inoculated bacteria could survive in the soil and around the roots, but their numbers declined progressively. Wani et al. (1988) reported that though the survival of Azospirillum in soil was very poor, the counts of inoculated Azospirillum could be increased by 2 to 3 fold by repeated inoculation for three years.

The establishment of Azospirillum spp. and Herbaspirillum strains marked with antibiotic resistance was investigated in a field experiment with rice (Pereira et al., 1988). Percentage establishment of the inoculated strains were determined at flowering stage and grain filling stage. Establishment of the inoculated strains on roots was observed only at flowering, A. lipoferum strains Al 121 isolated from rhizosphere soil of rice completely dominated the azospirilla population on washed roots; followed by A. brasilense Sp. 245; isolated from surface sterilized wheat

roots. Herbaspirillum seropedicae did not establish itself well.

2.10 EFFECT OF Azospirillum INOCULATION

2.10.1 Yield responses

Inoculation with A. lipoferum and A. brasilense isolated from rice roots and soils increased grain yields under green house and field conditions.

Subba Rao et al. (1979) reported that Azospirillum inoculation significantly increased the grain yield of rice variety pusa 2-21 at 0, 40 and 60 Kg N ha^{-1}.

Rinaudo et al.(1981) tested Azospirillum sp. isolated from rice and found differences in plant response to different isolates. Rhizosphere acetylene reduction activity appeared to be negatively correlated with the specific ARA of the strains inoculated in the rhizosphere. Strain 93, was the only one isolate proved significantly beneficial, probably because of its adaptation to soil.

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 N ha^{-1} of fertilizer N than at a higher level. Although the mean yields of both grain and straw were increased by inoculation with Azospirillum, the interaction

between N fertilizer and inoculation was not statistically significant.

Prasad and Singh (1984) reported the combined effect of inoculation of Azospirillum and incorporation of Azolla in rice in pot culture. Incorporation of Azolla as a green manure, inoculation of seedlings with Azospirillum and or application of N increased growth, nutrient uptake and rice yield.

Azospirillum inoculation significantly increased the filling rate of strains and grain yield per plant in rice (Watanabe and Lin, 1984). Kumar and Balasubramanian (1989) reported that 25 to 50 Kg N ha^{-1} can be saved by Azospirillum inoculation in rice cultivation.

Field experiments carried out in Tamil Nadu with rice (ADT 36) revealed that inoculation with A. brasilense increased grain yield up to 22% and straw yield by 8 to 41% (Gopalaswamy and Vidhyasekaran, 1987). The number of productive tillers, grain yield and straw yield in rice cultivars ADT 36, IR 50 and ADT 37 were also increased by inoculation of Azospirillum (Gopalaswamy and Vidhyasekaran, 1988).

Field experiments conducted for a period of seven years using rice, wheat, maize and barley showed that the

inoculated plants fertilized with reduced nitrogen levels ranging from 20 to 30 Kg ha^{-1} gave equivalent or slightly higher yields of the control field with full nitrogen fertilization (Favilli et al., 1987). Rao et al. (1987) reviewed the data on the effect of inoculation with N_2 fixing bacteria on rice yield and concluded that yield response to inoculation is most pronounced at moderate levels of (30-60 Kg N ha^{-1}) fertilizer nitrogen. Thus, inoculation with N_2 fixing bacteria combined with fertilizer N could increase yields.

Azospirillum inoculation was found to be beneficial for upland rice (Purushothaman, 1988). The grain and straw yields of direct sown rice were increased by seed treatment and soil application of Azospirillum (Gopalaswamy et al., 1989).

Balasubramanian and Kumar (1989) reported that seed treatment and soil application of Azospirillum increased total biomass and grain yield of a number of rice varieties.

Farmers can obtain higher yield in direct seeded rice that can later be converted into wet land rice by applying irrigation water at 45 days after sowing, Azospirillum inoculation and split applications of potassium fertilizer (Govindasamy et al. 1992).

Azospirillum inoculation alone or in combination with low levels of chemical N significantly increased grain and straw yield of rice (cv. Himdhan) in two consecutive years of study under the midhill condition of Himachal Pradesh (Sharma et al., 1992). Inoculation at nitrogen levels of 0, 30, and 60 Kg ha^{-1} gave yield equivalent to that obtained with nitrogen levels of 30, 60 and 90 Kg, respectively; showing an additive effect of 30 Kg N ha^{-1}.

Gunasekaran et al. (1992) reported an increased activity of glutamate synthase (GOGAT), glutamine synthetase (GS) and glutamate dehydrogenase due to Azospirillum inoculation in rice cultivar ponni. Inoculation also increased shoot and root length of rice plant.

2.10.2 Nitrogen fixation

The effects of inoculation are generally believed to be due to increased N_2 fixation and nitrogen input. However, lower or similar rates of N_2 fixation was reported in the rhizosphere of inoculated compared to control plants (Nayak et al., 1986; Rao and Rao, 1983). Van Berkum and Sloger (1983) reported with wild rice that declining carbon reserves and increasing levels of ammonium would almost certainly combine to restrict nitrogenase activity. Trolldenier (1987) estimated the contribution of associative N_2 fixation to the nutrition of low land rice in a long term

pot experiment with ten consecutive crops of rice. Low level nitrogen application favoured associative nitrogen fixation.

Ito et al. (1980) and Gowda and Watanabe (1985) using whole plant and excised plant, respectively also found higher $^{15}N_2$ incorporation in the outer leafsheath than in the root.

A temporary increase in nitrogenase activity in the rhizosphere occurred due to inoculation, but this increase became insignificant, as the rice plants grew older (Rao and Rao, 1983; Watanabe and Lin, 1984).

Studies on ARA, ^{15}N and N balance studies failed to prove emphatically that higher N_2 fixation is solely responsible for N_2 uptake (Nayak et al., 1986).

2.10.2.1 Varietal responses

Nitrogen fixation associated with rice plants involves several complex interactions between the rice plant, the microorganisms and the ecosystem. Several investigations suggest variation in the nitrogenase activity (acetylene reduction assay) associated with different rice varieties (Trolldenier, 1977; Lee et al., 1977; Rinaudo et al., 1981; Ladha et al., 1985; Rao and Rao, 1985; Ladha et al., 1986). Nitrogen fixing (acetylene reduction) activity associated with roots of 12 different rice varieties were assayed at heading stage by an in situ chamber acetylene reduction method. The in situ ARA and population of N_2 fixing bacteria

obtained on nitrogen free malate medium for Azospirillum spp. enrichment showed positive correlation (Kang et al., 1986).

Watanabe (1986) suggested that screening for rice varieties that greatly stimulate N_2 fixation would be the most efficient way of manipulating the bacterial association. Suitable methods are developed and /or are being developed for effective screening of rice varieties with higher N_2 fixation (Watanabe, 1986; Ladha et al.,1986). Results indicate that the capacity of a particular rice variety to stimulate N_2 fixation is associated with higher dry matter production and characters linked to photosynthesis (Ladha et al., 1986).

2.10.2.2 Transfer of fixed nitrogen to the rice plant

Although several studies indicate nitrogen fixation in rice ecosystem, only limited attempts were made to study the transfer of the fixed N_2 to the rice plant. When rice plants at heading were transplanted from the field to a culture solution and then exposed to $^{15}N_2$ for 7 days, less than 10% of the fixed N_2 was transferred to the leaf and panicles (Ito et al., 1980). In another experiment under green house conditions, Yoshida and Yoneyama (1980) exposed rice plants to $^{15}N_2$ for 7-13 days. These studies revealed the bulk of the fixed N_2 in the root zone soil and about 25% of the N_2

fixed in the rice rhizosphere was transferred to the rice plant. A major portion of the fixed nitrogen (80%) remained in the soil (Eskew et al., 1981). Ito and Watanabe (1981) clearly demonstrated that more nitrogen was available to the rice plant from the biologically fixed N than from the native soil N. These results suggest that a larger percentage of the fixed N_2 would be available for the successive crop than for the standing crop. In addition, heterotrophic nitrogen fixation by microorganisms in rice ecosystem offers a great potential source of fixed N_2.

2.10.3 Enhancing plant growth

The plant growth promoting influence of Azospirillum has been extensively examined (Michiels, 1989; Sumner, 1990). One of the earliest and best described events in Azospirillum root colonization was the impact on root development. Although,there were slight variations between different partnerships, the inoculated mature plants showed a more developed root system in all cases (Patriquin et al., 1983; Jain and Patriquin,1984, Kapulnik et al., 1985). Tien et al. (1979) proposed that changes in root morphology are caused by plant growth factors produced by Azospirillum. They found that inoculation of pearl millet greatly increased proliferation of lateral roots and root hairs.

Azospirillum inoculated roots of pearl millet and guinea grass produced more mucilaginous sheath, root hairs and

lateral roots than uninoculated control (Umali-Garcia et al., 1980). Nayak et al (1986) reported that inoculation of rice with Azospirillum increased the production of tillers, plant height and early reproductive growth. Muralidhara (1985) observed that cytokinins isolated form Azospirillum or kinetin did not influence the seedling growth for the first five days. But after 14 days the length of radical was higher in cytokinin treatments than that in the control. Treatment with cytokinins from A. lipoferum influenced radical length significantly. Plumule length was also higher but not statistically significant.

Purushothaman et al. (1987) observed increased root growth in rice seedlings inoculated with Azospirillum. Some of the recent research concerning the Azospirillum plant interaction has focussed on phytohormone production by these bacteria (Kucey, 1988; Bashan and Levanony, 1990)

2.10.3.1 Production of plant growth promoting substances

Reynders and Vlassak (1979) were the first to report that Azospirillum convert tryptophan to indole acetic acid. Tien et al (1979) reported the excretion of auxins particularly IAA, gibberellins and cytokinins by A. brasilense. They stated that the root exudates of plants contained tryptophan which probably acted as precursor for the synthesis of IAA.

Gaskins and Hubbell (1979) attributed the plant growth response observed after inoculation with Azospirillum to the production of plant growth promoting substances. Tien et al. (1979) reported that inoculation of pearlmillet with Azospirillum strains greatly increased proliferation of lateral roots and root hairs.

Horeman et al. (1986) found that IAA producing capacity of A. brasilense was more than that of A. lipoferum and no gibberellins were produced by both the species. Analytical characterization, by gas chromatography - mass spectrophotometry (GC-MS) of microbial GAs has been reported in cultures of Azospirillum lipoferum. (Bottini et al., 1989). Recently, Janzen et al. (1992) provided evidence from capillary gas chromatography - mass spectrometry analysis that pure cultures of A. brasilense can produce GAs in a chemically defined medium and also in co-culture with Trichoderma harzianum on moist barley straw.

Zimmer and Bothe (1988) gave an alternate explanation for the plant growth promotion by Azospirillum. Due to the nitrate respiration by this bacterium, nitrite is formed, which will react with a substance in the cell and the product formed (could be ascorbate) functions as an auxin. Hence they postulated that IAA and nitrite probably are the only factors causing an enhancement of the growth of roots of grasses.

Zimmer et al. (1991) reported the identification of a locus in A. brasilense, which enhanced Trp-dependent IAA production in A. irakense KA3, a strain producing low IAA.

2.10.4 Enhancing mineral uptake

Enhanced proliferation of the root system promote increased mineral uptake (N, P and K) and consequently increase the accumulation of dry matter (Lin et al., 1983; Okon 1985, Okon and Kapulnik,1986). In a hydroponic system under green house condition, inoculation of rice seedlings with Azospirillum resulted in enhanced PO_4^- ion uptake of the plants in 4 of the 7 samples tested (Murty and Ladha, 1988).

2.10.5 Stimulation of root exudation

Two strains of Azospirillum (A. lipoferum 4B and A. brasilense A.94) isolated from the rhizosphere of rice caused an increase in root exudation, when 3 day old seedlings grown on a C and N free medium in a closed vial were inoculated with 6×10^7 bacteria per plant and grown for one week (Heulin et al., 1987). Shoot carbon incorporation and respiration were reduced by inoculation. A third strain (A. brasilense RO 7) caused no significant change in exudation. A. lipoferum B7C isolated from maize did not stimulate root exudation.

2.10.6 Enhancing enzyme activity

Nitrate reductase activity of wheat roots was increased on inoculation of homologous strains of Azospirillum (Ferreira et al., 1987). Gunasekaran et al. (1992) reported increased activity of glutamate synthase (GoGAT), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) in rice cv. Ponni due to Azospirillum inoculation. Maximum enzyme activity was recorded on 60th day of inoculation.

2.10.7 Production of pectic enzymes

Very low pectinolytic activities were detected in extracts of wheat roots inoculated with Azospirillum which may be involved in slight hydrolysis of the middle lamellae of the Azospirillum colonized cortex cells without causing cell collapse, and may accelerate uptake of water and minerals by the roots (Lin et al., 1983; Kapulnik, et al., 1984, Sarig et al., 1984). A. irakense was found to produce pectate lyase and pectin methyl esterase activity and fix nitrogen when pectin was the sole carbon source (Khammas and Kaiser, 1991). The four other species of Azospirillum failed to show pectinolytic activity.

2.10.8 Physiological changes in plant roots

As a result of colonization by Azospirillum, certain physiological changes have been reported to occur in plant roots. In tomato seedlings the respiration rate per gram of

dry weight was shown to be lower in inoculated roots than in non inoculated controls (Hadas and Okon, 1987). This suggests that more dry material is accumulated with less energy in roots colonized with Azospirillum than in controls. They also observed that inoculation resulted in significant increase in the specific activity of malate dehydrogenase in tomato root tips. Other enzymes related to the tricarboxylic acid cycle, the glycolysis pathway, the synthesis of aromatic amino acids, and the breakdown of organic phosphate in maize roots also increased due to inoculation (Okon, 1987 quoted by Michiels et al., 1989). He also found that activation of phenylalanine - ammonia - lyase (PAL) and glucose - 6 - phosphate dehydrogenase enzymes, which are diagnostic for most bacterial and fungal infections, was not observed in Azospirillum colonized maize roots.

2.10.9 Oxygenation of rhizosphere

Plants like rice growing under submerged conditions develop cortical airspace called aerenchyma which facilitate atmospheric oxygen transport through the leaf aerenchyma to the roots by longitudinal diffusion inside the plant and by radial diffusion from the root airspace to the ectorhizosphere, so creating an O_2 gradient around the root. Ueckert et al. (1990) studied the effects of Azospirillum inoculation on radial gas diffusion from roots of rice.

Inoculation by A. brasilense Cd showed a striking 3-5 fold increase in permeability of root cell wall causing an enhanced oxidation of the rhizosphere. This may support colonization of roots by diazotrophs, which fix nitrogen under microaerobic conditions. The enhanced cell wall permeability may be probably due to IAA like phytohormones released by the bacteria.

2.11 FACTORS AFFECTING THE SUCCESS OF INOCULATION

Several factors influence the efficiency of the inoculated bacteria and N_2 fixation in the soil and rhizosphere.

2.11.1 Soil moisture

Soil moisture exerted a profound influence on $^{15}N_2$ incorporation in several rice soils. (Charyulu and Rao, 1979) Nitrogen fixation was low when the soil moisture content was below 25% (Rao, 1978). Yoshida and Ancajas (1973) demonstrated significant N_2 fixation in the rhizosphere of lowland but not of upland rice. The stimulatory and inhibitory effects of combined nitrogen varied greatly with respect to the moisture regime in several rice soil (Charyulu and Rao 1979). Nitrogenase activity increased several fold following a shift from nonflooded to flooded conditions, and decreased when the flooded soil was returned to non flooded conditions (Nayak and Rao 1981).

2.11.2 Soil pH

Charyulu and Rao (1979) investigated the rate and extent of N_2 fixation in four Indian rice soils - alluvial, laterite, acid sulphate (pokkali) and acid sandy saline (karapadam). Despite high acidity and salinity, appreciable nitrogen fixation was evident in pokkali and karapadam soils under both flooded and non flooded conditions.

Rao *et al.* (1978) reported the ubiquitous presence of nitrogen fixing *Spirillum* in different rice soil types of (Alluvial, laterite, pokkali and kari) under both upland and lowland conditions.

Dobereiner *et al.* (1976) reported pH as an important factor deciding the distribution of *Azospirillum*. A pH of around seven was found to be optimal, but sporadic occurrence was noticed in soils with pH 4.8. Surface sterilized roots of *Panicum maximum* harboured the diazotroph, irrespective of the pH of the soil from where it was collected.

Azospirillum spp. isolated from non saline rice soils exhibited higher nitrogenase activity than those isolates obtained from saline soils. Nitrogen fixation of *Azospirillum* decreased with an increase in the salinity level in the medium (Jena *et al.*, 1988).

Soil acidity factors (high Al and Mn and low pH and Ca) adversely affected the survival, growth and nitrogen fixation of microorganisms (Munns, 1977). Rai (1986) studied the manganese resistance of native Azospirillum. It showed a declining pattern of growth and N_2 fixation at and over 55 μg Mn ml^{-1} and ultimately stopped at 275 μg ml^{-1}. Nitrosoguanidine induced manganese resistant strains of A. brasilense were isolated and characterised for growth. Some of the mutant strains showed a good deal of cross resistance to Al^{3+} (660 μg Mn ml^{-1} + 2.5 μg Al ml^{-1}) and showed higher nitrogenase activity.

New and Kennedy (1989) studied the regional distribution and pH sensitivity of Azospirillum associated with wheat roots in Eastern Australia. Azospirillum was isolated from wheat roots and associated soil from 22 of 100 samples. Of the 88 wheat root samples for which soil pH was measured, azospirilla were not isolated from any of the 31 with soil pH below 4.5 and were infrequently isolated between pH 4.5 and 5.0. Strains isolated from soils had a minimum pH for growth that was less than the pH of the soil from which they were isolated. However, a significant proportion of strains isolated from roots had a minimum pH for growth that was higher than the pH of the associated soil suggesting that wheat roots provided an ecological niche protecting against soil acidity.

A. irakense was found to grow in a pH range of 5.5 to 8.5 (Khammas et al.. 1989). Sundaram et al. (1992) did the protoplast fusion of Azospirillum strains in order to genetically engineer superior strains. Protoplasts of Azospirillum sp. strain showing increased acid tolerance (pH 3.4) and A_2 204 showing increased nitrogen fixation were isolated and fused using PEG 6000 at 40% concentration.

2.11.3 *Organic matter*

Addition of rice straw, rice roots and green manure enhanced nitrogen fixation in some of the rice soils of Philippines (MacRae and Castro,1967). Rao (1978) reported that application of rice straw and other carbon sources greatly enhanced nitrogenase activity in several rice soils. Charyulu and Rao (1979) found that addition of cellulose to both flooded and non flooded soils enhanced nitrogen fixation. Negi et al . (1986) recorded higher population of Azospirillum in the rhizosphere of barley (Hordeum vulgare L.) grown in soils amended with organic matter, such as city compost and rice straw compost and the proliferation was more pronounced in inoculated plants.

In monoxenic systems, the numbers of A. brasilense were found to be positively correlated to soil organic matter content (Albrecht et al., 1981). Del Gallo and Fabbri (1991) reported that dual inoculation of Azospirillum and Rhizobium

improved nodulation of chickpea and this interaction was further enhanced by organic matter present in the growth medium.

The negative influences of phenolic compounds released from decomposing rice straw on nitrogen fixation by Rhizobium (Rice et al., 1981) indicated that the physiological capabilities of some rhizobacteria can be altered in the presence of complex substrates. However, Azospirillum grown along with a cellulose degrading bacterium Cellulomonas gelida in a cellulose medium exhibited growth and nitrogen fixation (Halsall and Gibson, 1985).

Growth of the cellulose degrading fungi, Trichoderma harzianum did not show any adverse effect on production of GA by Azospirillum, when both of them were co-cultured on moist barley straw. (Janzen et al., 1992).

2.11.4 Interactions with other microorganisms

Azospirillum in soil must live in a highly competitive environment where many interactions occur with different microbial groups. Kulinska and Drozdowicz (1983) studied 56 strains of streptomyces and 72 strains of fungi isolated from maize and rice rhizosphere for their antagonistic activity against A. lipoferum and A. brasilense. All streptomyces were antagonistic either to some or to all

indicator A. lipoferum. Antagonistic activity against A. brasilense was less common among streptomyces. Only 7 molds were able to inhibit growth of some azospirilla. The antagonistic activity of both streptomyces and fungi was attributed to their ability to produce antibiotic like factors.

Azospirillum grew and fixed nitrogen in co-culture with cellulose degrading bacteria (Halsall and Gibson, 1985). Janzen et al. (1992) inoculated A. brasilense and a cellulolytic fungus Trichoderma harzianum on moist barley straw. The fungus or the degradation products of straw had no adverse effect on A. brasilense. In this co-culture system also the bacteria produced gibberellins. There are also evidences to show the positive influence of Azospirillum on Rhizobium - legume symbioses (Sarig et al., 1986; Del Gallo and Fabbri, 1991).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The laboratory experiments were conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK Campus, Bangalore 560 065. The field experiment was conducted at the Agricultural Research Station, Kankanady, Mangalore. The materials used and methods followed are given below.

3.1 POPULATION OF *Azospirillum* IN RICE SOILS OF CERTAIN ACIDIC SOILS OF COASTAL KARNATAKA

Soil samples were collected from Mangalore, Brahmavar, Adyar and Kasargod for studying the distribution of azospirilla in acidic soils of coastal Karnataka. To determine the seasonal variation of the bacterial population samples were collected during the months of September 1990, December, 1990 and April 1991. Representative soil samples from upland, midland and low lands were collected from ARS, Kankanady Mangalore.

The upland represents single cropped rainfed rice lands. The plots classified under low lands had water availability during most of the period of the year and used for raising three crops of rice. In the midlands, two or three crops of rice can be grown depending on the availability of irrigation water.

Samples were taken at different depths, 0-10 cm, 11-20 cm and 21-30 cm and brought to the laboratory immediately. Following serial dilution techniques the total heterotrophic bacteria and Azospirillum were enumerated.

3.2 ENUMERATION OF Azospirillum

The MPN (Most Probable Number) technique was used for the enumeration of Azopirillum (Okon et al., 1977; Hegazi et al., 1979). The semisolid malate media was taken in test tubes. Formation of characteristic subsurface white pellicles in malate semisolid medium, a change to dark blue colour of the medium, positivity in acetylene reduction test (more than 5 n moles of C_2H_4 hr^{-1} $tube^{-1}$) the appearance of scarlet colonies when streaked on congored agar medium (Rodriguez-Cacere's, 1982) and the occurrence of typically curved cells on microscopical examination served as indices in the quantification of Azospirillum (Dobereiner et al., 1976). The most probable number of Azospirillum was calculated from the statistical tables of Cochran (1950) and expressed per gram dry weight of soil or root.

3.3 ISOLATION OF Azospirillum

Azospirillum was isolated from rice roots as well as soil.

The following cultivars or local collections of rice were used for the isolation of Azospirillum. The seeds were

obtained from the germplasm collection of the Agricultural Research Station, Kankanady.

1. Aluga
2. Athikara
3. Doddapatty
4. Doddi
5. Gandhasala
6. IR. 8-68
7. IR. 26
8. IR. 36
9. Jaya
10. Jyothi
11. Madhu
12. Mahavira
13. Mala
14. Nethravathi
15. Panama
16. Pushpa
17. Rashmi
18. Rajakayama and
19. Triveni

The seeds of all the genotypes were sown in pots filled with soil collected from rice fields of ARS, kankanady and sufficient number of plants were raised under unsterile

conditions. Isolation was attempted when the seedlings attained 30 days growth.

Azospirillum cultures were isolated from the roots of rice by following the enrichment culture technique as described by Day and Dobereiner (1976).

The seedlings were uprooted and the washed root bits were aseptically placed into 5 ml of the semisolid malate medium (NFb) (Baldani and Dobereiner, 1980) supplemented with 50 mg per liter of yeast extract. After 48 hr of incubation at 30°C, characteristic white pellicles of Azospirillum were observed just below the surface of the medium. Subsequently, loopfuls of cultures were transferred to nitrogen free malate medium, and observed just for the development of subsurface pellicles. From this tube a loopful of culture was streaked on NFb agar plates containing 50 mg yeast extract per litre. After one week, typical small white dense, single colonies were picked and transferred into semisolid NFb medium. Pellicle formation in this medium indicated the success of isolation. For final purification these cultures were streaked on to potato agar (BMS) (Baldani and Dobereiner, 1980) and the typical colonies were again tested on NFb medium supplemented with congored according to the method described by Rodriguez - Cacereus (1982).

Azospirillum was isolated from surface sterilized roots also. Rice roots were thoroughly washed, surface sterilised and crushed in a sterile mortar and suspended in sterile phosphate buffer saline. Dilutions of the crushed roots were inoculated into tubes containing 5 ml of NFb malate semisolid medium. Isolation of Azospirillum was done as described earlier.

Soil samples collected from rice fields were serially diluted and inoculated into 20 ml test tubes containing 5.0 ml of NFb malate semisolid medium. From the tubes showing positive for Azospirillum growth, isolation of the organism was done as described earlier.

3.4 IDENTIFICATION OF ISOLATES OF Azospirillum

The identification of Azospirillum in the enrichment culture in the semi-solid medium was accomplished by determining the presence of very characteristic, white, dense and undulating fine pellicles and higher nitrogenase activity. Shape, motility and presence of PHB granules were observed in 72 hr old cultures of the isolates upon microscopic observation. Gram staining was carried out as per Hucker's modification (Rangaswami, 1975).

3.4.1 Physiological tests

i) Acid from glucose (Tarrand et al., 1978)

The medium used had the following composition.

		g l^{-1}
Peptone	:	2.0
Ammonium sulphate	:	1.0
Magnesium sulphate	:	1.0
Ferric chloride	:	0.002
Manganese sulphate	:	0.002
Bromothymol blue	:	2.0 ml of 0.5 per cent alcoholic solution.

The pH was adjusted to 7.0-7.1 with potassium hydroxide. The medium was dispensed in 5.0 ml quantities in test tubes and sterilized. Filter sterilized glucose was added to a final concentration of 1.0 per cent.

48 hr old cultures from test tube slants were taken and suspended in sterile phosphate buffer saline and a quantity of 0.1 ml was inoculated into the medium. After a period of 4 days of incubation at 37°C, yellow colour indicated acid production.

3.4.2 Determination of the Carbon Sources

The nitrogen free semisolid malate medium (Baldani and Dobereiner, 1980) was prepared to test the ability of the isolates to use different carbon sources. Bromothymol blue

was omitted, malate was replaced by one per cent carbon sources (Glucose, Sucrose). The medium was inoculated from 4 hr. old cultures and the growth at 37°C was observed for 3-4 days after inoculation.

3.4.3 Requirement for Biotin

The nitrogen free malate medium (Baldani and Dobereiner, 1980) was used for assessing the requirement for biotin. Two sets of media were prepared with and without biotin (BDH, India) (0.0001 g l^{-1}). 48 hr old cultures grown in nutrient broth were centrifuged washed twice with sterile phosphate buffered saline and resuspended in water to a uniform density. A quantity of 0.1 ml of this suspension was used to inoculate 5.0 ml volume of medium and incubated for 48 hr at 37°C. In case where slight growth occurred in medium without biotin, a second serial transfer was made to confirm the biotin requirement.

3.4.4 Test for Dissimilation of Nitrate

Tests for nitrate reduction and denitrification were performed as described by Neyra et al. (1977). In the NFb medium, NH_4NO_3 was added (5 mM) and 10 ml of the medium were inoculated with a loopful of stock culture and incubated at 33°C for 60 hr. The cultures were then handshaken gently to mix the pellicle with the medium, and gas production and nitrite accumulation were recorded.

Nitrite was determined on 0.1 ml aliquots from the cultures by adding 2.0 ml of a 1:1 (v/v) solution of 0.02% (1-naphthyl)ethylene diamine hydrochloride and 1.0% sulfanilamide in 1.5 M HCl. Water was added to make a final volume of 4.0 ml and the colour was allowed to develop for 15 min. Development of a purple red colour indicated the presence of nitrite. Accumulation of NO_2 was evidence of NO_3 reduction, while disappearance of NO_2 from the medium was evidence for NO_2 reduction.

3.5 DETERMINATION OF INTRINSIC ANTIBIOTIC RESISTANCE OF *Azospirillum* ISOLATES

The level of intrinsic antibiotic resistance shown by the *Azopirillum* isolates against the following antibiotics was studied as per the method of Josey *et al*., (1979).

1. Streptomycin (Str), obtained as Ambistryn-s. Sarabhai Chemicals, Baroda.
2. Chloramphenicol (Cam) obtained from Parke Davis (manufacturing) Ltd. Calcutta.

The *Azospirillum* cultures were grown in nutrient broth under shake culture condition for 72 hr. A loopful of the standardized inoculum was streaked on to NFb medium (supplemented with 1.5 per cent agar and 50 mg yeast extract per litre) containing individually the antibiotics at different concentrations. The required amount of freshly prepared and filter sterilised antibiotic solutions were added to the medium after cooling down to 40°C to get the

required final concentration. The medium containing the respective antibiotic was dispensed in 20.0 ml quantities into sterile petri dishes and Azospirillum isolates were inoculated. The plates were incubated at 32°C for 48 hr and the growth observed. The results were reaffirmed by incorporating the antibiotics at different concentrations in NFb broth supplemented with 50 $\mu g\ l^{-1}$ yeast extract and inoculating the respective isolates of Azospirillum. Characteristic growth of Azospirillum was observed for resistant cultures. The natural level of resistance shown by the wild type of Azospirillum towards different antibiotics was calculated.

3.6 In vitro NITROGENASE ACTIVITY OF THE ISOLATES OF Azospirillum

The nitrogen free semisolid malate medium (Baldani and Dobereiner, 1980) was prepared in 5.0 ml quantities in test tubes. The medium was inoculated with a loopful of Azospirillum and incubated at 32°C in static condition. After 5 days of growth the cotton plugs were removed and replaced by tight fitting needle puncture rubber stoppers (suba-seals). 10.0% of the air from the tube was evacuated and 10.0% of pure acetylene gas was injected. The tubes were incubated at 32°C for 24 hr in static condition without disturbing the pellicle. Production of ethylene due to reduction of acetylene was measured using Shimadzu Gas chromatograph-7G fitted with flame ionization detector (FID)

with poropak-R column (length 3m, mesh size 80-100, carrier gas N_2 with a flow rate of 60 ml min^{-1}, column temperature 70°C). A quantity of 1.0 ml of the gas sample was withdrawn, through an air tight sterile disposable syringe and fed into the gas chromatograph. Peak length, attenuation and range were recorded. One ml of pure standard ethylene gas was injected and using this value, the nitrogenase activity of the samples were calculated. The nitrogenase activity was expressed as *n* moles of ethylene produced hr^{-1} mg $protein^{-1}$ (Hardy et al.,1968).

3.6.1 Estimation of the protein of the cells

To 3 ml of the homogenised culture taken in a tube, 6.0 ml of 10 per cent potassium hydroxide was added and boiled for 20 min, till the solution became clear. the solution was made up to 10.0 ml and 1.0 ml was used for the estimation of protein content by Lowry's method (Lowry et al.,1951).

3.6.1.1 Composition of the reagents

a. Alkaline copper solution.

A quantity of 50.0 ml of the reagent A was mixed with 10.0 ml of the reagent B at the time of use.

Reagent A : Two per cent sodium carbonate in 0.1 N sodium hydroxide solution.

Reagent B : Copper sulphate 0.5 per cent in 1.0 per cent sodium tartarate.

b. Folin-Ciocalteu reagent (IN)

One ml of the sample was taken in a test tube and distilled water was added to make up the volume to 4.5 ml. Five ml of the alkaline copper solution was added to each tube and mixed well and allowed to stand for 10 min. Then 0.5 ml of Folin-Ciocalteu reagent was added, mixed well and the intensity of blue colour developed was read at 660 nm against an approximate blank. The protein content was estimated by referring to a standard graph prepared with bovine serum albumin.

3.7 ACETYLENE REDUCING ACTIVITY OF ISOLATES OF Azospirillum OBTAINED FROM RICE CULTIVARS AS WELL AS ARA OF EXCISED ROOTS

Twelve cultivars/local entries of rice were grown in pots using soil from ARS, Kankanady. one month after growth, the ARA of the excised roots was determined and expressed as n moles g^{-1} root hr^{-1}.

The ARA of the isolates of Azospirillum obtained from surface sterilised/non surface sterilised roots of the above cultivars was also determined as described earlier.

3.8 RESISTANCE OF Azospirillum TO HEAVY METALS

NFb medium (Baldani and Dobereiner, 1980) supplemented with 0.1% yeast extract was used for the study. The medium was supplemented with different concentrations of Mn^{2+} (in

the form of $MnCl_2.4H_2O$), Al^{3+} (in the form of $KAl(SO_4)_2.12H_2O$ and Fe (in the form of Ferrous monosodium ethylene diamine tetra acetic acid). The above chemicals were filter sterilised and added to the sterilized medium to get the desired final concentration of the chemicals. The agar medium was poured into sterile petriplates at 20 ml $plate^{-1}$ and allowed to dry. Triplicate plates of these media were inoculated with a loopful of dilute suspension (10^4 to 10^5 cells ml^{-1}) of each isolate. Plates were incubated at 30^oC and examined for growth indicating their resistance to the particular heavy metal.

3.9 EFFECT OF HEAVY METALS ON GROWTH OF Azospirillum

Isolates which had shown higher resistance to the metal toxicity on agar plate screening experiments were used for this study. Different concentrations of filter sterilised solutions of Mn^{2+}, and Al^{3+} were incorporated into the sterilized NFb medium (Baldani and Dobereiner, 1980) and adjusted the pH to 6.5. Azospirilla were inoculated and the growth (optical density at 560 nm) was determined under microaerophilic conditions at 30 ± 1^oC after 3 and 5 days of incubation.

3.10 EFFECT OF LOW pH ON THE GROWTH OF Azospirillum

Five isolates of Azopirillum were selected for the study. NFb medium (Baldani and Dobereiner, 1980) with increased buffering capacity having 6.7 g malate, and 8.7 g

K_2HPO_4 (New and Kennedy, 1989) with different pH levels (4.0, 5.0, 5.5, 6.0, 6.5 and 7.0) were employed for the study. Two levels of $MnCl_2$ (0.002 ppm and 1.0 ppm) were supplemented to the medium. The selected isolates were inoculated and the growth (optical density at 560 nm) was determined under microaerophilic conditions at 30 $\pm$ 1°C after 72 hr of incubation.

3.11 EFFECT OF *Azospirillum* ON SEEDLING VIGOUR INDICES OF RICE

Dehulled seeds of rice variety sakthi were sterilised with 70% ethanol for 5 min and with 0.1 per cent sodium hypochlorite for 3 min and with acidified 0.1% mercuric chloride for 2 to 3 min. Each treatment was followed by several washings with sterile distilled water. Seeds were dipped in a suspension of *Azospirillum* (10^6 cells ml^{-1}) for a minimum of 6 hr. Seeds treated with sterile distilled water was used as control. One hundred seeds were used for each treatment with three replications. Germination test was conducted in accordance with the international rules for seed testing (Anonymous, 1976) by the modified roll towel method. After 5 days of growth the vigour indices of the seedlings were calculated in the following method proposed by Abdul-Baki and Anderson (1973).

Vigour index = Germination percentage X total length of seedlings.

3.12 SCREENING OF *Azospirillum* UNDER POT CULTURE CONDITIONS

The seeds of rice (cv. Sakthi) were surface sterilised as described earlier. The lignite based inoculum of different isolates of *Azospirillum* were prepared. Slurries of different inocula were made. Seeds were treated with different inocula, air dried for one hour and sown in plastic pots containing soil from ARS, Kankanady. Two plants were allowed per pot with three replications. One month after sowing shoot length, root length and biomass were recorded.

3.13 EFFECT OF *Azospirillum* INOCULATION AT GRADED LEVELS OF NITROGEN FERTILIZER ON RICE

A field experiment was laid out at ARS, Kankanady to determine the effect of *Azospirillum* inoculation on rice in the acidic laterite soil. The experiment was conducted during the kharif season of 1991, using the cultivar Sakthi. Treatments were as follows :

T_1 : Full N + *Azospirillum* inoculation

T_2 : Full N

T_3 : 75% N + inoculation

T_4 : 75% N

T_5 : 50% N + inoculation

T_6 : 50% N

T_7 : 25% N + inoculation

T_8 : 25% N

T_9 : No N + inoculation

T_{10} : No N, no inoculation.

The experiment was laid out in a completely randomized block design with three replications. The gross size of each plot was 3mx3m. Azospirillum was inoculated by seedling dip method (Rao et al., 1983). Twenty day old seedlings raised in a nursery were removed placed in trays filled with the aqueous slurry of lignite based Azospirillum for a period of 4-6 hr. Care was taken to immerse all the roots in the slurry sufficiently. The seedlings, inoculated and uninoculated, were transplanted and raised to maturity following recommended cultural practices. A fertilizer dose of N:P:K: 75:75:90 Kg ha^{-1} was given in three split doses as per the package of practice recommendation. Observations were recorded at periodic intervals.

Plant height, shoot dry weight and root dry weight were recorded one month and two months after transplanting and also at harvest. Total number of tillers, productive tillers, grain yield and straw yield were recorded at harvest. Nitrogen content of straw, grain and roots was determined. Populations of Azospirillum, heterotrophic nitrogen fixers and total heterotrophic bacteria were enumerated 1 month, 2 months and 3 months after transplanting.

Acetylene reducing activity of the excised roots was determined 60 days after transplanting and expressed as n moles of acetylene reduced g root^{-1} hr^{-1}.

3.14 DETERMINATION OF NITROGEN CONTENT OF SAMPLES

Samples of roots, straw and grain were dried in an oven at 70^{o}C to a constant weight and powdered in a grinder and used for analysis. The nitrogen content was estimated by Kjeldahl method (Jackson, 1973). Plant samples (0.5 g) were taken in digestion tubes and a spatula full of the digestion mixture consisting of potassium sulfate, copper sulfate and selenium in 100:10:1 proportion was added and 5 ml of concentrated sulphuric acid was poured. The tubes were kept for digestion at 250^{o}C for 30 min and 400^{o}C for one hour in a digestion unit (MRK, Quick Digester, Model QDS 10 M, MRK, Japan). The digestion was considered complete, when the solution became clear.

The digested sample was then distilled using Kjelmatic auto vapor - still (Model VS-FA-1), Mitamura, Riken, Japan, where the digested sample was mixed with 25 ml of 40 per cent sodium hydroxide and the liberated ammonia was trapped into 25 ml of 4 per cent boric acid to which four drops of mixed indicator of methyl red and methylene blue was added. The indicator was purple initially but after ammonia was trapped, it turned to green colour. This was

titrated against 0.05 N sulphuric acid until original purple colour was obtained.

3.15 ENUMERATION OF THE NON-RHIZOSPHERE, RHIZOSPHERE AND ENDORHIZOSPHERE MICROORGANISMS

Nonrhizosphere, rhizosphere and endorhizosphere samples were collected (Watanabe et al., 1979) and the population of Azospirillum, heterotrophic bacteria and dinitrogen fixers were enumerated. Rice plants were gently removed from the soil and soil adhering to roots was removed under water. The removed soil from the final washing was used as the rhizosphere soil sample.

The roots were cut into 3-5 cm pieces and washed with sterile water six times and then macerated. This macerated tissue was used as histosphere samples.

Population of heterotrophs and N_2 fixers were enumerated by dilution plate method using soil extract agar medium (Rangaswami, 1966) and combined carbon medium (Rennie, 1981) respectively. Azospirillum was enumerated following serial dilution and MPN method.

3.16 STUDIES ON Chromobacterium

While studying the heterotrophic bacteria in the root environments of rice cv.Sakthi grown in the rice fields of ARS, Kankanady violet coloured bacterial colonies were frequently observed.

3.16.1 Tentative identification of the bacterium

The bacterium was identified as per the Bergey's Manual of Determinative Bacteriology, 1984. The following characteristics were studied. Colony characteristics on solid nutrient agar (NA) medium, Gram reaction, cell shape, motility, cell size, production of poly β -hydroxy butyrate, growth at different temperature, HCN production, arginine hydrolysis, acid and gas production from glucose, fructose and trehalose, gelatin liquefaction, casein hydrolysis, chitin digestion, agar digestion, urease production, methylene blue reaction and utilisation of citrate, L.alanine, L.glutamate, L.histidine, L.lysine, L.ornithine and L.phenyl alanine as sole carbon sources.

Following the standard procedures (Sneath, 1960), the pigment produced by the bacterium was identified. The tests conducted were

i. solubility in carbon disulfide, diethyl ether, amyl alcohol, methanol, glycerol, aniline, pyridine and dimethyl formamide.

ii. reaction with chlorine water, bromine water, H_2O_2, chromate and permanganate.

iii. treating ethanolic solution of the pigment with 10% KOH, 50% HNO_2, 50% H_2SO_4, glacial acetic acid, 2 M HCl, 0.1 M $FeCl_3$, and Acetic acid + Zn dust.

iv. absorption maximum of the pigment in ethanolic solution.

Based on the results, the bacterium was identified as Chromobacterium (Sneath, 1960;1984).

3.16.2 Occurrence of Chromobacterium in the root environments

The population of Chromobacterium present in the rice root environments was determined by serial dilution and plating on nutrient agar media. The total number of violet pigmented colonies were counted and expressed as number of colony forming units g^{-1} soil or g^{-1} root on dry weight basis.

3.16.3 Establishment of Chromobacterium in the root environment

Seeds of rice cv.Sakthi were surface sterilized as described earlier and inoculated with a suspension of Chromobacterium (containing 10^6 cells ml^{-1}). Another set of seeds were inoculated without surface sterilization. The germinated seeds were sown in unsterile soil and grown for 14 days. The seedlings were uprooted and establishment of the bacterium was studied in the rhizosphere, rhizoplane and histosphere by serial dilution and plating on nutrient agar medium. The population was expressed as number of colony forming units g^{-1} soil or root on dry weight basis.

3.17. *STUDIES ON* Bacillus sp.

An unusual type of bacterial colony was frequently observed in the nutrient agar plates used for studying heterotrophic bacteria from the roots of rice cv. Sakthi, collected from rice fields of ARS, Kankanady.

3.17.1 Tentative identification of the bacterium

The bacterium was isolated and purified. Tentative identification was attempted as per Bergey's Manual of Determinative Bacteriology, 1984.

The following characteristics were studied. Colony characteristics on nutrient agar medium, cell shape, motility, diameter, spore formation, Gram-reaction, aerobic growth, catalase activity, acidification of glucose, formation of indole, growth in 2% NaCl, and utilisation of lactate, glucose, malate, mannitol, pyruvate and succinate were studied. Based on the results the bacterium was identified as Bacillus sp.

3.17.2 Occurrence of Bacillus sp. in the root environment

The natural abundance of the bacterium was determined by dilution plating of the rhizosphere and histosphere samples of the rice roots cv.Sakthi. Population was expressed as number of colony forming units g^{-1} soil or root on dry weight basis.

3.17.3 Establishment of Bacillus sp. in the root environment

Seeds of rice cv.Sakthi were surface sterilised as described earlier and inoculated with a suspension of the bacteria (containing 10^6 cells ml^{-1}). Another set of seeds were inoculated without surface sterilization. The seeds were germinated and sown in unsterile soil and grown for 16 days. The seedlings were uprooted and establishment of the bacterium was studied in the rhizosphere and histosphere by serial dilution and plating on nutrient agar medium. The population was expressed as number of colony forming units g^{-1} soil or root on dry weight basis.

3.17.4 Acetylene reduction activity

Bacillus sp. was found to grow in semisolid nitrogen free medium (Baldani and Dobereiner, 1980). To determine their nitrogen fixing ability, a loopful of inoculum was introduced to the above medium (5 ml) and incubated at $30^{o}C$ for 4 days. Acetylene reduction activity was determined as described previously and expressed in n moles of ethylene reduced $tube^{-1}$ hr^{-1}.

3.17.5 Effect of inoculation of Bacillus sp. on vigour index of rice seedlings

Dehulled seeds of rice cv.sakthi were sterilised with ethanol, sodium hypochlorite and 0.1% mercuric chloride as described earlier. After washing thoroughly seeds were

inoculated with a suspension of Bacillus sp. (10^6 cells ml^{-1}) and dipped in the suspension for 6 hr. Another set was treated in the same way with cell free supernatant. The bacterial culture grown in nutrient broth for 72 hr was centrifuged at 8000 G for 15 min and obtained the supernatant. The pellets were washed thoroughly and resuspended in phosphate buffer and used for inoculation. Germination test was conducted as per the international rules for seed testing (Anonymous, 1976) by the modified roll towel method. After 6 days of growth the vigour indices of seedlings were calculated as per the method proposed by Abdul-Baki and Anderson (1973).

3.18 INTERACTION BETWEEN CERTAIN SOIL MICROORGANISMS AND *Azospirillum*

Pseudomonas fluorescences, *chromobacterium*, *Bacillus* sp. and *Azospirillum* (five isolates) were used for the study. Inhibitory effects of the above bacteria on *Azospirillum* or *vice versa* were studied on nutrient agar medium by cross streak method.

3.19 SURVIVAL OF *Azospirillum* IN DIFFERENT CARRIERS

Azospirillum was grown in NFb medium supplemented with 0.1% ammonium chloride. After 72 hr of growth, it was used for preparing carrier based inoculants. The carrier materials used for the study included lignite, perlite and soil + FYM. Apart from that alginate beads were also studied.

3.19.1 Preparation of inoculants

The carrier materials were dried, well powdered and sieved through 100 mesh size sieve. They were sterilized by autoclaving at 121°C for 15 min for 3 consecutive days. The sterility of the carrier material was checked by inoculating it into a nutrient medium.

The culture broth was mixed with the sterilized carrier aseptically to give approximately 1 x 10^7 cells g^{-1} uniformly in all treatments. The required quantity of calcium carbonate to bring the pH of the carrier material to 6.8 was added to broth culture before mixing with the carrier material. Soon after mixing, the carrier based cultures were packed into packets containing 200 gauge (50 µ) material polyethylene bags and sealed. The sealed packets were incubated for one week at room temperature before start of the experiment.

3.19.2 Encapsulation of Azospirillum in alginate matrix

The alginate-perlite beads were prepared as per Hegde and Brahmaprakash (1992). Azospirillum was grown as detailed above for 72 hr. Two g of sodium alginate and 2.0 g of perlite were added to 100 ml of broth, stirring thoroughly. The suspension was taken in a Pasteur pipette with a 1.0 mm - diameter orifice and dripped into a solution of 0.25 M $CaCl_2$. The pellets were recovered from the solution washed

with sterile distilled water and kept in sterile NFb medium for 24 hr. Pellets were collected, washed and dried overnight in a laminar air flow hood. They were stored under room conditions as well as at different temperatures and the viable populations were determined periodically.

3.19.3 Determination of viable number of *Azospirillum* in the carrier materials and alginate pellets

Survival of *Azospirillum* in the carrier materials and alginate pellets stored at $4^{o}C$, $10^{o}C$, $30^{o}C$ and $40^{o}C$ were determined. Representative samples were with drawn at 0, 15, 30, 60, 90 and 120 days of incubation and the number of colony forming units of *Azospirillum* in the samples were determined by serial dilution and plating on NFb medium suplemented with 100 mg l^{-1} yeast extract. The plates were incubated at $30^{o}C$ for 3-5 days. Three replications were maintained for each treatment in all the experiments. Since the alginate pellets were insoluble in water, they were disintegrated in a mixture of 8.7×10^{-2} M KH_2PO_4 and 3.0×10^{-2} M Na_2HPO_4 (pH 7.7) (Fravel *et al.*, 1985) and the *Azospirillum* population was assayed by dilution plating.

3.20 EFFECT OF SOIL MOISTURE ON THE SURVIVAL OF *Azospirillum*

Effect of four levels of moisture on the survival of *Azospirillum* in soil was studied. Soil was collected from ARS, Kankanady, dried and sieved through 2 mm sieve. It was sterilised by autoclaving at $121^{o}C$ for 15 min for three

consecutive days. Azospirillum was added as described earlier and final moisture levels (25%, 50% and 100% of field capacity) were adjusted with sterile distilled water. One set was kept at submerged condition by providing a standing water of 2 cm above the soil level; kept in a 250 ml Ehrlenmeyer flask. Four replications were maintained and samples were drawn at 0, 15, 30, 60, 90 and 120 days interval. Population of Azospirillum was determined as described earlier.

3.21 EFFECT OF STORAGE TEMPERATURE ON SURVIVAL OF Azospirillum IN SOIL

The survival of Azospirillum was studied in soil stored under four storage temperatures ($4^{o}C$, $10^{o}C$, $30^{o}C$ and $40^{o}C$). Three day old broth culture of Azospirillum was added to sterilized soil. The moisture content was adjusted to field capacity and packed in polyethylene bags as described earlier. The population of Azospirillum was determined at different periods of storage (viz., 0, 15, 30, 60, 90 and 120 days).

3.22 EFFECT OF ORGANIC MANURE, OIL CAKES AND NITROGEN ON POPULATION OF Azospirillum

The effect of farm yard manure, neem cake and groundnut cake on the population of Azospirillum was studied at different nitrogen and moisture levels. The different treatments are given below :

T_1 : FYM, No N, Field capacity

T_2 : FYM, No N, Submergence

T_3 : Neem cake, No N, Field capacity

T_4 : Neem cake, No N, Submergence

T_5 : Groundnut cake, No N, Field capacity

T_6 : Groundnut cake, No N, Submergence

T_7 : No organic manure, No N, Field capacity

T_8 : No organic manure, No N, Submergence

T_9 : FYM, 75% N, Field capacity

T_{10} : FYM, 75% N, submergence

T_{11} : Neem cake, 75% N, Field capacity

T_{12} : Neem cake, 75% N, Submergence

T_{13} : Groundnut cake, 75% N, Field capacity

T_{14} : Groundnut cake, 75% N, Submergence

T_{15} : No organic manure, 75% N, Field capacity

T_{16} : No organic manure, 75% N, Submergence

Soil was collected from rice fields of ARS Kankanady, dried in shade and sieved. 100 g quantity of the soil was taken in 250 ml flask and kept for incubation after covering it tightly. After bringing the moisture levels to the desired extent, organic manures were applied at 10 t. ha^{-1}. For keeping the soil under submerged condition, a standing water of 5 cm was maintained above the soil level. After 10 days of incubation at $30^{o}C$, fertilizer N was added @ 100 kg N ha^{-1} as urea as per the treatment. This was to simulate

the field conditions where fertilizers are applied after 10 days of organic manure incorporation. The experiment was designed in CRD with 16 treatments and three replications. Samples were withdrawn at regular intervals and population of Azospirillum and heterotrophic bacteria were enumerated and expressed as number of colony forming units per g dry soil.

3.23 STATISTICAL ANALYSIS

To test the significance of the effect due to different treatments, the experimental data were statistically analysed. The field experiment was laid out in a randomized block design (RBD) and the in vitro studies were carried out in completely randomized design (CRD). The statistical analysis was carried out by computing the analysis of variance as suggested by Panse and Sukhatme (1961).

RESULTS

IV. RESULTS

4.1 POPULATION OF *Azospirillum* IN THE ACIDIC SOILS OF COASTAL KARNATAKA

The population of *Azospirillum* in the upland soils of Kankanady was maximum during September (5.07×10^5 cell g^{-1} soil) and minimum during April (4.53×10^3) (Table 1, Fig.1). The low land and midland had stagnant water during September. During April the soil was dry. In these soils population was maximum during December (Lowland : 117.9×10^5; Midland 15.60×10^5) and minimum during April (Lowland 14.03×10^3; Midland 11.0×10^3).

The top 10 cm soil recorded higher population during September and December, while the deeper layers of 20 cm and 30 cm recorded higher counts during April. In the upland soil the *Azospirillum* count in September was 5.15×10^5 and 4.2×10^5 at 20 cm and 30 cm depth, respectively. The population decreased to 1.3×10^5 and 1.8×10^5 in December and recorded a minimum of 13.1×10^3 and 8.4×10^3 during April at the two depths.

Similar trend was observed in low land and midland. The MPN counts of *Azospirillum* in the low land soils at 20 cm and 30 cm depths were 3.24×10^5 and 1.42×10^5 during September, 123×10^5 and 92×10^5 during December and 20×10^3 and 16×10^3 during April. The population of *Azospirillum* in

Table 1. Population of Azospirillum and heterotrophic bacteria in certain acidic soils of Karnataka and Kerala in different seasons ($10^x g^{-1}$ dry soil)

Season	September 1990			December 1990			April 1991		
Location	Azospirillum	Heterotrophs	percent Azospirillum	Azospirillum	Heterotrophs	percent Azospirillum	Azospirillum	Heterotrophs	percent Azospirillum
1	x 10^5 2	x 10^6 3	4	x 10^5 5	x 10^6 6	7	x 10^3 8	x 10^6 9	10
I Non Saline Soils									
A. Kankanady									
i) Upland									
10 Cm	5.85	51.60	1.13	4.90	180.30	0.27	4.10	10.80	0.04
20 Cm	5.15	60.00	0.86	1.30	105.30	0.12	13.10	16.80	0.08
30 Cm	4.20	43.00	0.98	1.80	59.00	0.31	8.40	4.30	0.20
Mean	5.07	38.63		2.67	114.87		8.53	10.63	
ii) Lowland									
10 Cm	4.65	31.30	1.49	136.00	260.00	5.23	6.10	15.30	0.04
20 Cm	3.24	42.60	0.76	123.00	318.00	3.87	20.00	42.00	0.05
30 Cm	1.42	30.60	0.46	92.00	210.00	4.38	16.00	35.00	0.05
Mean	3.10	34.83		117.00	262.67		14.03	30.77	
iii) Midland									
10 Cm	4.31	27.50	1.57	16.00	360.00	0.44	8.00	9.00	0.09
20 Cm	3.53	20.50	1.72	16.30	410.00	0.40	14.00	15.00	0.09
30 Cm	3.04	15.4	1.97	14.50	305.00	0.48	11.00	11.00	0.10
Mean	3.63	21.13		15.60	358.33		11.00	11.67	
B. Brahmavar									
10 Cm	3.50	62.70	0.56	7.80	84.00	0.93	21.00	3.00	0.70
20 Cm	2.40	58.30	0.41	5.00	73.00	0.69	63.00	25.00	0.25
30 Cm	1.50	38.40	0.39	1.60	62.00	0.26	52.00	20.00	0.26
Mean	2.47	53.13		4.80	73.00		45.33	16.00	
C. Kasargod									
10 Cm	7.97	886.70	0.09	23.00	140.30	1.64	15.00	40.20	0.04
20 Cm	6.84	781.00	0.09	17.00	95.60	0.18	45.00	60.10	0.08
30 Cm	6.40	77.30	0.83	15.00	90.70	1.65	30.00	51.20	0.06
Mean	7.07	581.67		18.33	108.87		30.00	50.50	

Table 1. (continued...)

1	2	3	4	5	6	7	8	9	10
II Saline Soils									
a) Adyar									
10 Cm	8.40	40.30	2.08	14.40	90.00	0.16	1.10	4.00	0.028
20 Cm	3.20	36.20	0.88	8.40	81.00	1.04	2.00	20.00	0.010
30 Cm	1.40	25.20	0.56	7.60	68.00	1.12	0.50	14.00	0.004
Mean	4.33	33.90		10.13	79.67		1.20	12.67	
b) Kasargod									
10 Cm	4.80	97.40	0.49	2.14	85.00	0.25	3.00	25.00	0.012
20 Cm	3.40	86.40	0.39	3.50	73.00	0.48	5.00	31.00	0.016
30 Cm	3.10	73.10	0.42	1.20	75.00	0.16	4.50	33.00	0.014
Mean	3.77	85.63		2.28	77.67		4.17	29.67	

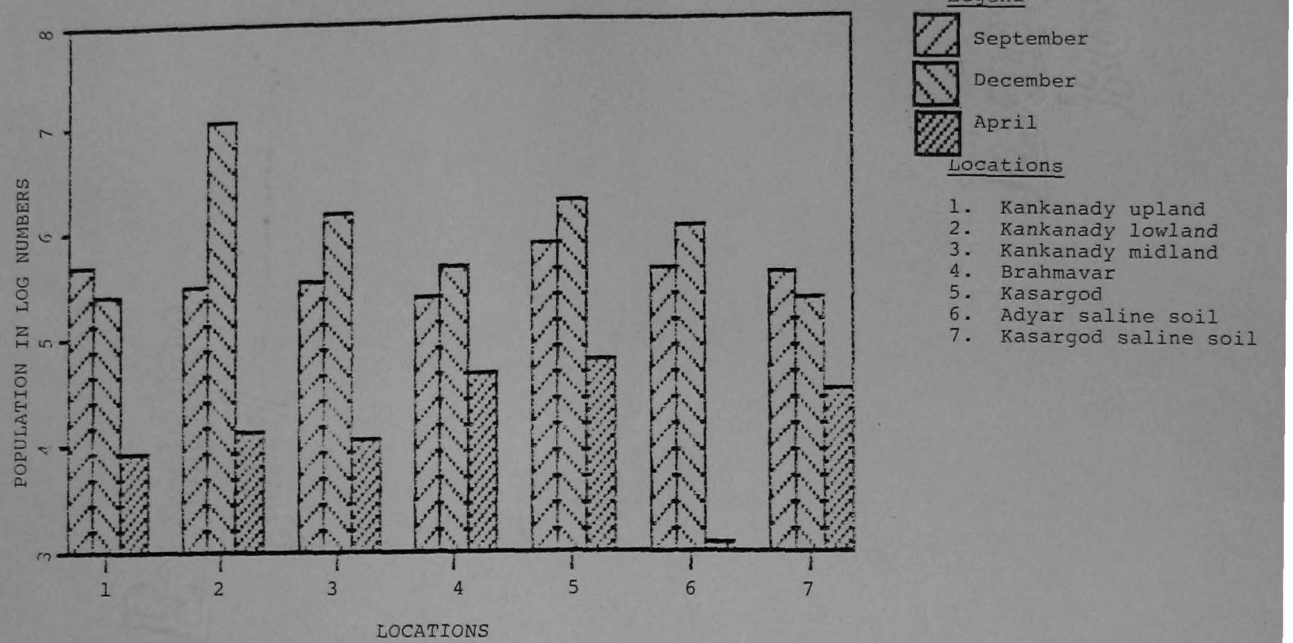


FIG.1. POPULATION OF Azospirillum IN CERTAIN COASTAL ACIDIC SOILS IN DIFFERENT SEASONS.

the midland soil at 20 cm and 30 cm depth was 3.53×10^5 and 3.04×10^5 during September; 16.3×10^5 and 14.5×10^5 during December and 14×10^3 and 11×10^3 during April.

Total heterotrophic bacteria recorded was maximum during December and Minimum during April (Table 1, Fig 2). In the upland soils of Kankanady the population of total bacteria at 10 cm, 20 cm and 30 cm soil were 51.6×10^6, 60.0×10^6 and 43.0×10^6 during september 180.3×10^6, 105.3×10^6 and 59.0×10^6 during December and 10.8×10^6, 16.8×10^6 and 4.3×10^6 during April. In the midland soils the cfu of heterotrophic bacteria were 27.5×10^6, 20.5×10^6 and 15.4×10^6 during September; 360×10^6, 410×10^6 and 305×10^6 during December and 9×10^6, 15×10^6 and 11×10^6 during April at depths of 10 cm, 20 cm and 30 cm respectively. In low land soils collected from depths of 10 cm, 20 cm and 30 cm, the counts of total heterotrophs were 31.3×10^6 and 210×10^6 during December and 15.3×10^6, 42×10^6 and 35×10^6 during April.

Population of Azospirillum and total heterotrophs were enumerated in the soils collected from Brahmavar. During September and December, the top 10 cm layer of soil harboured more population of Azospirillum, while it was more at 20 cm depth during April. Maximum counts of the bacterium was recorded in December (7.8×10^5), while it was reduced

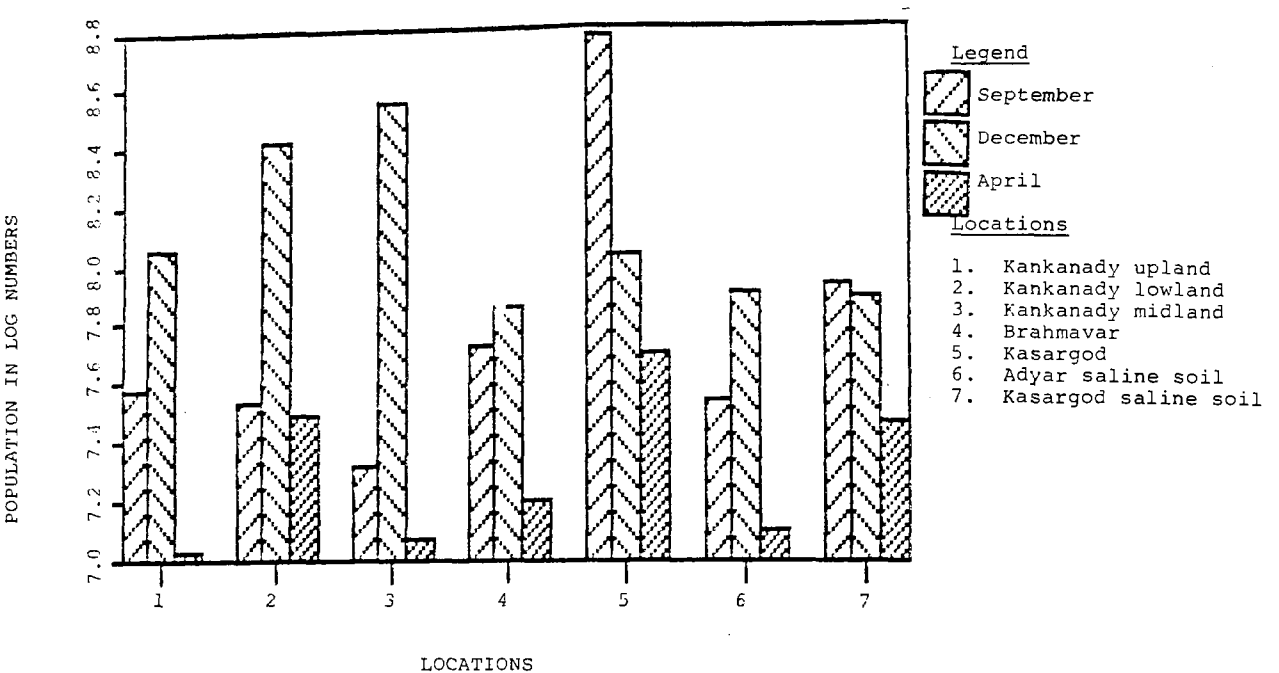


FIG.2. POPULATION OF HETEROTROPHIC BACTERIA IN CERTAIN COASTAL ACIDIC SOILS IN DIFFERENT SEASONS

to 21 x 10^3 in April, at 10 cm depth. The population at 20 cm and 30 cm were 2.4 x 10^5 and 1.5 x 10^5 in September; 5.0 x 10^5 and 1.6 x 10^5 in December and 63 x 10^3 and 52 x 10^3 in April.

Highest count of total heterotrophs was obtained in December (73 x 10^6) and lowest in April (16.0 x 10^6). During September and December, the upper 10 cm layer had more population (62.7 x 10^6 and 84 x 10^6, respectively) compared to the lower levels. Cfu of heterotrophs at 20 cm and 30 cm depths were 58.3 x 10^6 and 38.4 x 10^6 in September, 73.0 x 10^6 and 62.0 x 10^6 in December, and 25.0 x 10^6 and 20.0 x 10^6 in April.

Soil samples were collected from 10 cm, 20 cm and 30 cm depths from Kasargod and the population of Azospirillum and total bacteria were enumerated. A maximum MPN count of Azospirillum (23 x 10^5) was recorded in December and a minimum of 15 x 10^3 in April at 10 cm depth. The soils of upper 10 cm had higher numbers of Azospirillum during September and December, while it was lower during April. The MPN counts of Azospirillum in the soils of 20 cm and 30 cm depths were 6.84 x 10^5 and 6.4 x 10^5 during September, 17 x 10^5 and 15.0 x 10^5 during December; and 45 x 10^5 and 30 x 10^5 during April. Total heterotrophic bacteria was more in September (886.7 x 10^6) and least in April (40.2 x 10^6) in the 10 cm soil. Bacterial counts at 20 cm and 30 cm depths

were 781 x 10^6 and 77.3 x 10^6, respectively during September, 95.6 x 10^6 and 90.7 x 10^6, respectively during December and 60.1 x 10^6 and 51.2 x 10^6, respectively during April.

The saline acidic soils of Kasargod and Adyar showed fairly good population of the diazotroph, Azospirillum in the soils collected from Adyar, the maximum count (14.4 x 10^5) of Azospirillum was recorded during December and minimum during April (1.1 x 10^3) in the 10 cm top soil. At 20 cm and 30cm depths the population of the bacterium was found to be 3.2 x 10^5 and 1.4 x 10^5 in September 8.4 x 10^5 and 7.6 x 10^5 in December and 2.0 x 10^3 and 0.5 x 10^3 in April respectively . In the saline soils of Kasargod, the maximum count of Azospirillum was obtained during September (4.8 x 10^5) at the top 10 cm layer. The minimum number in the top layer was recorded in April (3.0 x 10^3). At 20 cm and 30 cm depths, the soil population of Azospirillum was 3.4 x 10^5 and 3.1 x 10^5 during September; 3.5 x 10^5 and 1.2 x 10^5 during December and 5.0 x 10^3 and 4.5 x 10^3 during April.

The counts of heterotrophic bacteria in these saline soils of Adyar showed a maximum of 90 x 10^6 in the top 10 cm layer during December, and a minimum of 4.0 x 10^6 during April. The population in the lower depths of 20 cm and 30 cm were 36.2 x 10^6 and 25.2 x 10^6, respectively in September,

81.0 x 10^6 and 68 x 10^6 respectively in December and 20 x 10^6 and 14 x 10^6 respectively during April. The saline soil from Kasargod recorded a maximum of 97.4 x 10^6 Cfu of heterotrophs during September in the top 10 cm layer which was reduced to 25 x 10^6 during April. The counts of bacteria at lower layers of soil viz. 20 cm and 30 cm were 86.4 x 10^6 during September, 73.0 x 10^6 and 75.0 x 10^6 during December and 31.0 x 10^6 and 33 x 10^6 during April.

In the soils of Kankanady, Azospirillum represented only a small percentage of the total heterotrophic bacteria. It was maximum during September (1.13%) in the upper 10 cm; which was drastically reduced to 0.038% in April. The trend was similar in the low land and midland also. In the lowland, maximum percentage of Azospirillum was observed in December, where it represented 4.38% of the total heterotrophs of the 21-30 cm depth. In the midland, the highest percentage recorded was 1.97% at 30 cm depth during September.

In the soil samples of Brahmavar, the highest percentage of Azospirillum to the heterotrophs was 0.93% observed during December in the top 10 cm soil and least of 0.25% during April.

Azospirillum constituted a maximum of 2.08% of the heterotrophs during September in the saline soil of Adyar.

The lowest figure of 0.03% was seen during April. In the saline soil collected from Kasargod, Azospirillum accounted for a maximum of 0.49% in September and 0.01% in April.

4.2 ISOLATION OF Azospirillum

By enrichment culture technique, Azospirillum isolates were obtained from soil/root tissues of rice. These isolates exhibited characteristic dense white undulating subsurface pellicle, about 2 mm below the agar surface. The colour of the medium changed from initial blue green colour to brilliant blue colour.

When the isolates were streaked on nitrogen free malate agar plates containing 50 mg yeast extract per litre, typical white dense single colonies appeared. The colonies appeared on potato dextrose (BMS) plates were pink, often wrinkled in appearance. In nutrient agar medium, the colonies were opaque, glistening raised and with lobed edges. These characteristics confirmed the description of Azospirillum.

The source of the isolates is presented in Table 2. Eighty eight isolates were obtained and subjected to various tests to fix up their identity (Table 3).

4.2.1 Microscopic Examination

Microscopic examination revealed the presence of typical fat droplets (PHB granules) within the cells , and

Table 2. Sources of *Azospirillum* isolates

Location/rice Cultivar	Description	Isolate Number
A. Soil samples		
1) Brahmavar	pH 5.3	Os 1 to Os 10
2) Kankanady	pH 5.8	Os 11 to Os 20
3) Kasargod	pH 5.4	Os 81 to Os 89
4) Adyar	pH 5.3,saline	Os 71 to Os 74
5) Kasargod	pH 5.3,saline	Os 75 to Os 78
B. Roots of rice cultivars /local entries		
1) Doddapatty	NSS	Os 41
	SSR	Os 24
2) Rajakayama	NSS	Os 42
	SSR	Os 21
3) Mahavira	NSS	Os 43
	SSR	Os 22
4) Jyothi	NSS	Os 44
	SSR	Os 23
5) Athikare	NSS	Os 45
	SSR	Os 25
6) Panama	NSS	Os 46
	SSR	Os 28
7) Aluga	NSS	Os 47
8) Gandhasala	NSS	Os 48
	SSR	Os 29
9) Nethravathi	NSS	Os 49
	SSR	Os 30
10) Jaya	NSS	Os 50
	SSR	Os 31
11) Mala	NSS	Os 51
	SSR	Os 32
12) IR 26	NSS	Os 52
	SSR	Os 27
13) IR 8-68	NSS	Os 53
	SSR	Os 26
14) Triveni	NSS	Os 54
	SSR	Os 33
15) IR - 36	NSS	Os 55
	SSR	Os 34
Saline resistant varieties		
16) Pushpa	NSS	Os 90 to Os 95
17) Madhu	NSS	Os 96 to Os 101
18) Rashi	NSS	Os 103 to Os 107
19) Doddi	NSS	Os 108 to Os 111

NSS : Roots were not surface sterilised
SSR : Roots were surface sterilised

active spiral movement. All the isolates were Gram negative in reaction.

4.2.2 Production of acid from glucose peptone broth

Certain isolates were found to produce acid from glucose peptone broth, a characteristic of A.lipoferum. The intensity of acid production was found to vary depending upon the isolate. Those that acidified glucose were considered A.lipoferum.

4.2.3 Utilization of different carbon sources

Malate, succinate, and lactate were found to be acceptable carbon sources for growth and nitrogen fixation by all the isolates, whereas glucose, mannitol and -Ketoglutaric acid were used only by a group of isolates mostly characteristic of A.lipoferum. A.brasilense was characterised by its inability to utilize glucose and mannitol. A.amazonense utilized glucose but not mannitol, while A.halopraeferens could not utilise glucose, but showed weak growth on mannitol.

4.2.4 Requirement of Biotin for Growth

Semisolid malate medium supplemented with biotin favoured the growth of all isolates. However, medium without biotin supported the growth of A.brasilense and A.amazonense, while A.lipoferum and halopraeferens required biotin for growth.

Table 3. Biochemical characteristics of the isolates of Azospirillum

1 Isolate No.	2 Growth in Congo red medium	3 Motility	4 Gram reaction	5 Presence of PHB	6 Pleomorphism on alkaline medium	Utilization of carbon sources: 7 Malate	8 Succinate	9 Lactate	10 Glucose	11 Keto-glutarate	12 Mannitol	13 Sucrose	14 Acidification of glucose peptone broth	15 Biotin requirements	16 Nitrate reductase	17 Denitrification	18 Tentative identification
Os 1	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 2	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 3	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 4	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 5	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 6	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 7	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 8	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 9	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	L
Os 10	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 11	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	L
Os 12	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 13	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 14	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 15	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	B
Os 16	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Cs 17	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 18	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 19	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 20	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 21	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 22	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 23	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 24	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 25	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 26	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 27	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	L
Os 28	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B

Table 3. (continued ...)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Os 29	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 30	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 31	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 32	+	+	-	+	-	+	+	+	+	+	-	-	-	-	+	-	U
Os 33	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 34	+	+	-	+	-	+	+	+	+	+	+	-	-	-	+	-	U
Os 41	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 42	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 43	+	+	-	+	+	+	+	+	+	+	-	-	-	-	+	+	U
Os 44	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 45	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 46	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 47	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 48	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 49	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 50	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 51	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 52	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 53	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 54	+	+	-	+	-	+	+	+	+	+	-	-	-	-	+	+	U
Os 55	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 71	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 72	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 73	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 74	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 75	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 76	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 77	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 78	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 81	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 82	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	L

Table 3. (Continued ...)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Os 83	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 84	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 85	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 86	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 87	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 88	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 89	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 90	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 91	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 92	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 93	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 94	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 95	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 96	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 97	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 98	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 99	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	B
Os 100	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 101	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 102	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	J
Os 103	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	L
Os 104	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	U
Os 105	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 106	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	L
Os 107	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	U
Os 108	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 109	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B
Os 110	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	L
Os 111	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	+	B

+ = Positive ; - = Negative ; B = Azospirillum brasilense
L = A. lipoferum; U = Uncertain identity

4.2.5 Denitrification property

All of the isolates utilised nitrate for their growth and reduced NO_3 to NO_2. Most of the isolates were also found to be capable of denitrifying. 19.32% of the isolates were non-denitrifers (Nir^-).

4.2.6 Salt tolerance

None of the isolates tested was found to be tolerant to 2.5% sodium chloride concentration. At lower levels of sodium chloride, the isolates were able to grow.

4.2.7 Tentative identification of the isolates

Based on the biochemical characteristics, the isolates were tentatively identified. Most of the isolates were A.brasilense (52.28%). A.lipoferum represented only 27.27% of the total 88 Azospirilla isolated. Eighteen isolates were showing properties which were not typical of the species identified so far including A.amazonense; A.halopraeferens or A.irakense. Distribution of Azospirillum spp. in samples collected from different sources are given in Table 4. Among the isolates obtained from non-saline soils, A.lipoferum constituted 10.23% and A.brasilense 20.45% of the total 88 isolates. The saline soils of Kasargod and Adyar yielded 4.55% of A. lipoferum and 3.41% of A.brasilense. The isolates obtained from nonsurface sterilised roots of rice seedlings had 3.41% of A.lipoferum and 6.82% of A.brasilense. Among the isolates obtained from

Table 4. Per cent distribution of *Azospirillum* spp. in different soils/root samples

Source	*A. lipoferum*	*A. brasilense*	Unidentified isolates
A. Soil (non saline)			
1) Brahmavar	1.14	10.23	-
2) Kankanady	6.82	3.41	1.14
3) Kasargod	3.41	6.82	5.68
Subtotal	11.37	20.46	6.82
B. Soil (saline)			
1) Adyar	2.27	1.14	1.14
2) Kasargod	2.27	2.27	-
Subtotal	4.54	3.41	1.14
C. Roots			
1) Not surface Sterilised	2.27	7.95	1.14
2) Surface sterilised	9.09	20.45	11.36
Subtotal	11.36	28.40	12.50
Total	27.27	52.28	20.46

Data represent distribution of 88 isolates of *Azospirillum*

surface sterilised roots of rice 9.1% were A.lipoferum and 69.22% A.brasilense.

4.3 ACETYLENE REDUCTION ACTIVITY OF THE Azospirillum ISOLATES

The isolates varied considerably in their ability to fix nitrogen, offering possibilities for exploiting selection of efficient nitrogen fixers (Table 5).

Based on the source of the isolates, the distribution pattern of efficient isolates revealed that the surface sterilized roots harboured more efficient isolates than the soil. Among the isolates obtained from surface sterilised roots, 83.87% showed ARA of above 100 n moles mg protein^{-1}, and 19.35% showed about 200 n moles mg protein^{-1}; while among the soil isolates only 18.18% showed ARA of above 100 n moles and only 6.06% showed above 200 n moles. The isolates OS 99 showed maximum nitrogenase activity (315.3 n moles mg protein^{-1} hr^{-1}).

4.3.1 ARA of excised roots of Azospirillum isolates obtained from rice cultivars

ARA of the excised roots of twelve cultivars of rice revealed wide variation with respect to their nitrogenase activity. The rice cv. panama showed a higher activity of 223.4 n moles g^{-1} hr^{-1} compared to a lowest activity of 67.4 n moles g^{-1} hr^{-1} in the cultivar Mahavir (Table 7).

Table 5. Acetylene reduction activity of the isolates of Azospirillum

Sl.No	Isolate	ARA*	Sl.No	Isolate	ARA	Sl.No	Isolate	ARA
1.	Os 1	10.2	27	Os 33	129.0	53	Os 83	12.1
2.	Os 3	28.6	28	Os 34	117.0	54	Os 84	47.2
3.	Os 4	163.3	29	Os 41	231.1	55	Os 85	21.3
4.	Os 5	120.1	30	Os 42	110.3	56	Os 86	18.9
5.	Os 7	45.6	31	Os 43	15.1	57	Os 87	34.2
6.	Os 8	210.4	32	Os 44	78.2	58	Os 88	25.0
7.	Os 9	79.2	33	Os 45	65.8	59	Os 90	151.2
8.	Os 10	63.01	34	Os 46	121.5	60	Os 91	163.7
9.	Os 11	48.8	35	Os 47	38.0	61	Os 92	147.3
10.	Os 13	123.1	36	Os 48	201.3	62	Os 93	204.2
11.	Os 14	96.2	37	Os 49	150.2	63	Os 94	187.3
12.	Os 15	88.1	38	Os 50	36.4	64	Os 95	115.3
13.	Os 17	6.0	39	Os 51	106.0	65	Os 96	88.9
14.	Os 18	34.0	40	Os 52	92.1	66	Os 97	7.5
15.	Os 19	21.0	41	Os 53	89.6	67	Os 98	17.3
16.	Os 20	15.0	42	Os 55	86.2	68	Os 99	315.3
17.	Os 21	157.0	43	Os 71	55.0	69	Os100	15.1
18.	Os 22	201.0	44	Os 72	34.0	70	Os101	116.3
19.	Os 23	210.0	45	Os 73	28.0	71	Os102	104.3
20.	Os 25	14.2	46	Os 74	57.0	72	Os103	110.2
21.	Os 26	195.1	47	Os 75	68.0	73	Os106	161.2
22.	Os 27	266.1	48	Os 76	94.0	74	Os108	121.5
23.	Os 28	234.2	49	Os 77	110.0	75	Os109	141.3
24.	Os 29	109.6	50	Os 78	163.1	76	Os110	189.2
25	Os 30	178.0	51	Os 81	86.3	77	Os111	133.2
26	Os 31	164.0	52	Os 82	71.2			

Data represent mean of three replications.

* n moles of C_2H_4 mg protein^{-1} hr^{-1}.

Table 6. Distribution of nitrogen fixing efficiency among isolates of *Azospirillum* spp. in soils/roots

Source of *Azospirillum* isolates	Mean ARA*	Percentage of total isolates possessing >100 n moles of ARA
I Soil		
a) Non saline	61.20	5.2
1. Brahmavar	90.05	3.9
2. Kankanady	54.03	1.3
3. Kasargod	39.53	0.0
b) Saline	76.14	5.2
1. Adyar	43.50	2.6
2. Kasargod	108.78	2.6
II Roots		
a) Not surface sterilised	101.56	7.8
b) Surface sterilised	147.84	33.8
1. From non saline soils	164.60	14.3
2. From saline soils	131.07	19.5

* n moles of C_2H_4 produced mg Protein^{-1} hr^{-1}.

Table 7. ARA of excised roots and Azospirillum isolates obtained from rice cultivars

	Cultivar	ARA of excised roots**	ARA of isolates obtained from Surface sterilised roots*	non-surface sterilised roots
1.	Athikare	102.10	65.30	14.2
2.	BR.51	148.30	169.30	129.0
3.	Gandhasala	116.50	201.30	109.6
4.	Jaya	90.90	136.40	164.0
5.	IR 26	110.50	92.07	265.1
6.	IR 36	89.30	86.20	117.0
7.	Jyothi	175.30	78.20	210.0
8.	Mahavira	167.40	15.05	201.0
9.	Mala	96.70	58.20	151.0
10.	Nethravathi	183.01	150.20	178.0
11.	Panama	223.40	121.50	234.0
12.	Rajakayama	155.30	110.30	157.0

* n moles of ethylene produced mg Protein^{-1} hr^{-1}.

** n moles of ethylene produced g^{-1} dry root hr^{-1}.

Isolates of Azospirillum obtained from the surface sterilised as well as non-surface sterilised roots also showed variation with respect to their nitrogen fixing ability.

ARA of Azospirillum isolate from the non-surface sterilised roots of the cultivar Nethravathi had an activity of 150.2 while that from the rice cv.Mahavira showed only 15.05. ARA of isolates obtained from surface sterilised roots also showed wide variation, ranging from 14.2 (cv.Athikara) to 265.1 (cv.IR26).

4.4 EFFECT OF SEED INOCULATION OF RICE WITH Azospirillum ON THE VIGOUR INDEX OF SEEDLINGS

On seed inoculation, most of the Azospirillum isolates increased the vigour of rice seedlings (Table 8, Plate 1). The maximum vigour index was recorded with the isolate OS77 (26.28% increase over distilled water). Twenty one isolates showed more than 10% increase in vigour index over the control. The increase in sprout length was up to a maximum of 23.89% (isolate OS106), the increase in root length was up to 29.16% (isolate OS10). Treatment with 16 isolates failed to enhance the vigour index. It can be seen that all the isolates enhanced shoot length. But the 16 isolates which did not enhance vigour index caused reduction in root length due to deformation of the roots. This

PLATE 1. Effect of Azospirillum inoculation on vigour index of rice seedlings

+A : Inoculated with Azospirillum

-A : No inoculation.

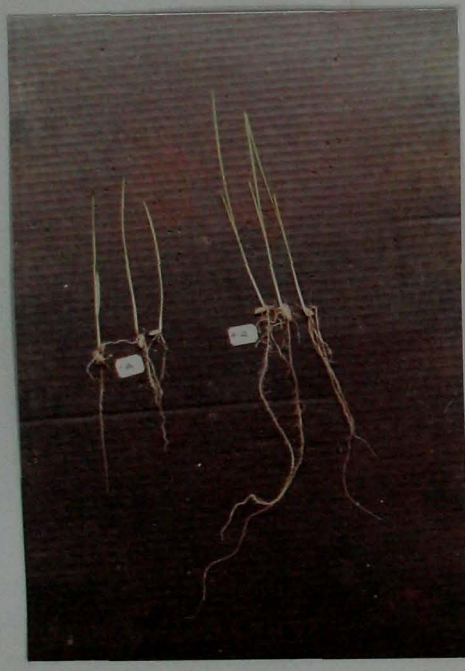


Table 8. Vigour Index* of rice seedlings$ as influenced by seed inoculation with different isolates of Azospirillum

Sl. No.	Isolate No.	Root length (cm)	Per cent increase over control	Shoot length (cm)	Per cent increase over control	Vigour index	Per cent increase over control
1	2	3	4	5	6	7	8
1.	Os 4	13.13	-1.57	6.37	13.55	1950	2.90
2.	Os 5	14.22	6.60	6.01	7.13	2023	6.76
3.	Os 7	16.94	26.99	6.82	21.57	2376	25.38
4.	Os 9	15.57	16.72	6.61	17.83	2218	17.04
5.	Os 10	17.23	29.16	6.21	10.70	2344	23.69
6.	Os 13	13.32	-0.15	6.12	9.09	1944	2.59
7.	Os 14	9.23	-30.81	6.12	9.09	1535	-19.00
8.	Os 15	12.01	-9.97	6.21	10.70	1822	-3.85
9.	Os 21	16.03	20.16	5.99	6.77	2202	16.20
10.	Os 22	14.61	9.52	6.52	16.22	2113	11.50
11.	Os 23	13.83	3.67	6.31	12.48	2014	6.28
12.	Os 25	15.82	18.59	6.25	11.41	2207	16.44
13.	Os 27	10.31	-22.71	5.92	5.53	1623	-14.35
14.	Os 28	12.64	-5.25	6.21	10.70	1885	-0.53
15.	Os 29	13.72	2.85	6.11	8.91	1983	4.64
16.	Os 30	14.03	5.17	6.31	12.48	2034	7.34
17.	Os 31	11.01	-17.47	6.27	11.76	1728	-8.81
18.	Os 33	10.88	-18.44	6.23	11.05	1711	-9.71
19.	Os 34	15.63	17.16	6.50	15.86	2213	16.78
20.	Os 41	11.01	-17.47	6.21	10.70	1722	-9.13
21.	Os 42	15.12	13.34	6.36	13.37	2148	13.35
22.	Os 44	16.12	20.83	6.75	20.32	2287	20.69
23.	Os 46	14.43	8.17	6.12	9.09	2055	8.44
24.	Os 48	13.12	-1.65	5.95	6.06	1907	0.63
25.	Os 49	15.93	19.42	6.31	12.48	2224	17.36

Table 8. (continued...)

1	2	3	4	5	6	7	8
26.	Os 51	11.31	-15.22	6.11	8.91	1742	-8.07
27.	Os 52	12.07	-9.52	6.21	10.70	1828	-3.53
28.	Os 53	13.22	-0.90	6.61	17.83	1983	4.64
29.	Os 55	14.86	11.39	6.42	14.44	2128	12.30
30.	Os 71	15.73	17.92	6.52	16.22	2225	17.41
31.	Os 73	11.52	13.64	6.33	12.83	1785	-5.80
32.	Os 75	14.91	11.77	6.27	11.76	2118	11.77
33.	Os 76	16.44	23.24	6.81	21.39	2325	22.69
34.	Os 77	17.12	28.34	6.81	21.39	2393	26.28
35.	Os 78	14.48	8.55	6.62	18.00	2110	11.35
36.	Os 81	15.58	16.79	6.52	16.22	2210	16.62
37.	Os 87	11.31	-15.22	6.62	18.00	1793	-5.38
38.	Os 90	13.68	2.55	6.26	11.59	1994	5.22
39.	Os 91	16.77	25.71	6.63	18.18	2340	23.42
40.	Os 93	8.64	-35.23	6.71	19.60	1535	-19.0
41.	Os 94	11.51	-13.72	5.67	1.07	1718	-9.34
42.	Os 97	9.42	-29.39	6.71	19.61	1613	-14.88
43.	Os 99	15.81	18.51	6.61	17.83	2242	18.31
44.	Os 101	10.51	-21.21	6.46	15.15	1697	-10.45
45.	Os 102	15.72	17.84	6.51	16.04	2223	17.31
46.	Os 103	9.05	-32.15	6.35	13.19	1540	-18.73
47.	Os 106	15.28	14.54	6.95	23.89	2223	17.31
48.	Os 109	13.42	0.60	6.17	9.98	1959	3.38
49.	Os 110	15.31	14.77	5.99	6.77	2130	12.40
50.	control	13.34	-	5.61	-	1895	-

* Vigour index = Germination percentage x total length of seedlings
$ cv. Sakthi

resulted in decreased the vigor index. The effect of inoculation was more pronounced on root development.

Based on acetylene reducing activity and influence on vigour index, the following 25 isolates were selected for further studies (OS7, OS10, OS21, OS25, OS34, OS41, OS42, OS46, OS49, OS53, OS55, OS58, OS71, OS75, OS77, OS81, OS87, OS90, OS91, OS93, OS99, OS102, OS106, OS109, and OS110).

4.5 PRELIMINARY SCREENING OF *Azospirillum* ISOLATES UNDER POT CULTURE CONDITION

Twenty five isolates were tested for their effect on plant growth under pot culture conditions (Table 9). Various isolates differed in their ability to bring about changes in plant growth parameters. Except one, all other isolates favoured growth. Maximum length of seedling (root length + shoot length) was observed in plants inoculated with isolates OS 99 (40.4 cm). These plants also showed maximum biomass yield (78.3% increase over control). Hence isolate OS 99 was selected for the field experiment. Inoculation with the isolate OS 41 which decreased the vigour index, resulted in less biomass production and root length of the seedlings compared to uninoculated control.

4.6 TOLERANCE OF ISOLATES OF *Azospirillum* TO HEAVY METALS

Thirty one isolates of *Azospirillum* selected for the study showed varying degrees of tolerance to the heavy

Table 9. Response of rice cv. Sakthi seedlings to inoculation with different *Azospirillum* isolates : Preliminary screening

Sl. No.	Isolate No.	Shoot length (cm)	Root length (cm)	Biomass (mg)	Per cent increase over control	
					Total Length	Biomass
1.	Os 7	24.75	6.73	88.6	19.74	24.26
2.	Os 10	23.98	7.12	84.1	18.30	17.95
3.	Os 21	30.20	7.6	10.3	43.80	44.50
4.	Os 25	25.20	6.9	84.7	22.10	18.80
5.	Os 34	27.30	6.8	84.5	29.70	18.50
6.	Os 41	18.91	7.01	64.6	-1.41	-9.40
7.	Os 42	26.47	8.12	108.1	31.60	51.60
8.	Os 46	21.85	6.74	99.3	8.75	39.30
9.	Os 49	24.62	7.11	78.7	20.70	10.40
10.	Os 53	26.90	6.20	83.9	25.90	17.70
11.	Os 55	19.81	7.40	98.3	3.50	37.90
12.	Os 58	28.88	7.31	107.0	37.70	50.07
13.	Os 71	29.20	6.90	97.5	37.30	36.75
14.	Os 75	21.78	6.70	85.0	8.30	19.20
15.	Os 77	25.60	8.61	116.6	30.10	63.50
16.	Os 81	25.85	7.36	85.1	26.30	19.40
17.	Os 87	23.19	7.10	81.7	15.20	14.60
18.	Os 90	28.25	6.81	98.4	33.40	38.00
19.	Os 91	25.50	6.30	95.1	21.00	33.40
20.	Os 93	24.50	5.80	97.1	15.30	36.20
21.	Os 99	30.61	9.81	127.1	51.97	78.30
22.	Os 102	25.12	6.20	78.7	19.10	10.40
23.	Os 106	27.53	7.30	102.9	32.50	44.30
24.	Os 109	24.58	8.10	94.3	24.30	32.30
25.	Os 110	26.98	5.90	93.3	25.10	30.90
26.	control	20.51	5.98	71.3	-	-

Data recorded 30 days after growth

metals aluminium ($AlCl_3$), manganese ($MnCl_2$) and iron (FeEDTA) (Table 10).

Nearly 90% of the isolates showed tolerance to 10 mM concentration of $AlCl_3$, while it was only 74.2% towards 20 mM and 35.5% towards 100 mM concentrations. Isolates obtained from saline soils of Kasargod and Adyar showed higher tolerance (Table 10). At 100 mM concentration, the percentage of isolats which showed growth was 100% and 33.3% respectively. While none of the isolates from Kasargod (non-saline) and Brahmavar (non-saline) showed tolerance to 100 mM concentration.

None of the isolates showed tolerance to manganese at 100 mM concentration. The percentage of isolates tolerant to 5 mM, 10 mM and 50 mM levels were 58.1%, 29.0% and 9.7% respectively. Fifty per cent of the isolates from Kasargod (saline soil) and 33.3% of the isolates from Kankanady were able to tolerate 50 mM concentration of $MnCl_2$. These isolates when grown at higher concentration of $MnCl_2$ showed a brown pigmentation which diffused into the growth medium.

Most of the isolates showed fairly high level of tolerance to Fe EDTA. About 43% of the total isolates were tolerant to 400 mM FeEDTA, while 6.45% showed tolerance even at 1000 mM level. About 43 per cent of isolates obtained from non-surface sterilised roots showed tolerance to 100 mM

Table 10. Resistance of *Azospirillum* isolates to heavy metals

Sl.No.	Isolate	Concentrations of Aluminium (mM)				Manganese (mM)				Fe (ppm)			
		5	10	20	100	5	10	50	100	50	100	400	1000
1.	Os 7	+	+	-	-	++	-	-	-	+	+	-	-
2.	Os 10	+	-	-	-	-	-	-		+	+	-	-
3.	Os 13	+	+	+	+	+	+	-	-	++	+	-	-
4.	Os 14	+	+	+	-	+	-	-	-	+	+	-	-
5.	Os 18	+	-	-	-	-	-	-	-	+	+	-	-
6.	Os 27	+	+	+	+	-	-	-	-	+	+	-	-
7.	Os 34	++	+	+	-	+	+	-	-	+	+	+	-
8.	Os 41	+	+	+	+	-	-	-	-	+	+	+	-
9.	Os 42	+	+	+	+	+	+	-	-	+	+	-	-
10.	Os 49	+	+	+	+	-	-	-	-	++	+	-	-
11.	Os 51	+	+	+	-	++	+	-	-	+	+	+	-
12.	Os 52	+	+	-	-	-	-	-	-	+	+	-	-
13.	Os 53	+	+	+	-	-	-	-	-	+	+	+	-
14.	Os 54	+	+	-	-	++	+	-	-	+	+	-	-
15.	Os 71	+	+	+	-	-	-	-	-	+	+	+	-
16.	Os 73	+	+	-	-	+	-	-	-	+	+	-	-
17.	Os 74	++	+	+	+	-	-	-	-	+	+	-	-
18.	Os 77	+++	+++	+	+	++	+	+	-	+++	+	-	-
19.	Os 78	+	+	+	+	-	-	-	-	+	+	+	+
20.	Os 88	++	+	+	-	++	-	-	-	+	+	-	-
21.	Os 89	+	+	+	-	-	-	-	-	+	+	+	+
22.	Os 90	+	-	-	-	-	-	-	-	+	+	+	-
23.	Os 91	++	+	+	-	+	-	-	-	+	+	+	-
24.	Os 94	+	+	+	-	-	-	-	-	+	+	+	-
25.	Os 97	++	+	+	-	++	+	+	-	+	+	-	-
26.	Os 99	++	+	-	-	++	+	-	-	+	+	-	-
27.	Os 101	+	+	+	+	++	-	-	-	++	-	-	-
28.	Os 102	+++	+	+	+	++	-	-	-	++	-	-	-
29.	Os 103	+	+	+	-	-	-	-	-	+	-	-	-
30.	Os 105	+	+	+	-	-	-	-	-	+	-	-	-
31.	Os 106	++	++	+	+	++	-	-	-	++	+	-	-

\+ = Presence of growth
\- = Absence of growth

Table 11. Distribution of Azospirillum isolates resistant to heavy metals

Source of isolates	No. of isolates tested	Percent of isolates showing resistance												
		Al concentrations (mM)				Mn concentrations (mM)					Fe concentrations (ppm)			
		5	10	20	100	<5	5	10	50	100	50	100	400	1000
A. Soil (non saline)														
1. Brahmavar	2	100	50.0	-	-	100	50.0	-	-	-	100	100	-	-
2. Kankanady	4	100	66.7	66.7		100	66.7	33.3	33.3	-	100	100	-	-
3. Kasargod	2	100	100	100	-	100	50	-	-	-	100	100	50	50
B. Soil (Saline)														
1. Adyar	3	100	100	66.7	33.3	100	33.3	-	-	-	100	100	33.3	-
2. Kasargod	2	100	100	100	100	100	50	50	50	-	100	100	50	50
C. Root														
1. Surface sterilised	12	100	75	83.3	33.3	100	58.3	25	8.3	-	100	66.7	33.3	-
2. Non-surface sterilised	7	100	100	71.4	42.9	100	42.9	42.9	-	-	100	100	42.9	-

concentration of $AlCl_3$ while only 33.3% of those obtained from surface sterilised roots showed tolerance to this concentration. However 8.3% of the isolates obtained from surface sterilised roots tolerated 50 mM concentration of $MnCl_2$, while none of those isolates obtained from nonsurface sterilised roots were tolerant to this concentration.

Increasing levels of $AlCl_3$ in the medium, adversely affected the growth of Azospirillum (Table 12). Among the different isolates tested OS99 showed better growth at 20 ppm level of $AlCl_3$ (OD.1:1), while OS 51 recorded the least growth (OD 0.65). Interestingly OS 99 also showed better growth compared to other isolates, at higher levels of $MnCl_2$.

The isolates which were able to tolerate higher levels of $MnCl_2$, showed slow growth at higher concentrations of this heavy metal (Table 13, Fig 3 & 4). Among the five isolates tested, all of them showed declined growth beyond 1.0 mm concentration of $MnCl_2$. However,the isolate OS77 was found to be less sensitive to Mn^{++} toxicity.

The effect of pH, monitored by tris buffer system, on the growth of Azospirillum was determined in N free batch culture after 3 days of incubation by determining the optical density at 560 nm (Table 14). pH below 6.0 was detrimental to the growth of the organism both in the

Table.12 Effect of aluminium on growth of Azospirillum isolates

Al^{3} Conc. (ppm)	Growth after 72 h (OD Values)					Growth after 110 h (OD Values)				
	OS42	OS51	OS54	OS77	OS99	OS42	OS51	OS54	OS77	OS99
5	0.9	1.1	0.8	0.9	1.0	0.9	1.2	1.0	1.1	1.2
10	0.8	1.0	0.5	0.7	0.8	0.85	1.1	0.7	0.8	0.9
20	0.3	0.1	0.2	0.1	0.5	0.5	0.11	0.4	0.4	0.6
Control	1.21	1.25	1.26	1.25	1.18	1.24	1.28	1.31	1.4	1.32

Table 13. Effect of manganese on growth of Azospirillum isolates

Sl. No	Mn^{++} Concentrations (ppm)	Isolates of Azospirillum				
		Os 42	Os 51	Os 54	Os 77	Os 99
A. Growth after 72 h. (O.D Values)						
1.	0.002	1.21	1.25	1.26	1.25	1.18
2.	1.000	0.92	0.90	0.82	1.10	0.91
3.	3.000	0.48	0.52	0.61	0.70	0.63
4.	5.000	0.13	0.16	0.18	0.16	0.28
B. Growth after 110 h. (O.D values)						
1.	0.002	1.24	1.28	1.31	1.40	1.32
2.	1.000	1.11	1.01	1.07	0.89	0.92
3.	3.000	0.53	0.57	0.68	0.76	0.71
4.	5.000	0.2	0.19	0.25	0.26	0.31

Table 14. Effect of p^H at two concentrations of Mn on the growth (OD values) of Azospirillum

pH	Concentrations of Mn^{++} (ppm)	Isolates				
		Os 42	Os 51	Os 54	Os 77	Os 99
4.0	0.002	0.20	0.30	0.21	0.25	0.21
4.5	0.002	0.30	0.30	0.40	0.35	0.31
5.0	0.002	0.38	0.36	0.42	0.38	0.37
5.5	0.002	0.43	0.46	0.52	0.61	0.61
6.0	0.002	0.71	0.73	0.68	0.72	0.71
6.5	0.002	0.87	0.85	0.86	0.86	0.84
7.0	0.002	0.91	0.92	0.92	0.94	0.94
4.0	1.0	0.15	0.18	0.16	0.17	0.20
4.5	1.0	0.26	0.28	0.31	0.27	0.28
5.0	1.0	0.33	0.32	0.38	0.31	0.29
5.5	1.0	0.38	0.36	0.44	0.41	0.38
6.0	1.0	0.52	0.58	0.48	0.48	0.51
6.5	1.0	0.71	0.70	0.64	0.66	0.68
7.0	1.0	0.78	0.73	0.68	0.69	0.72

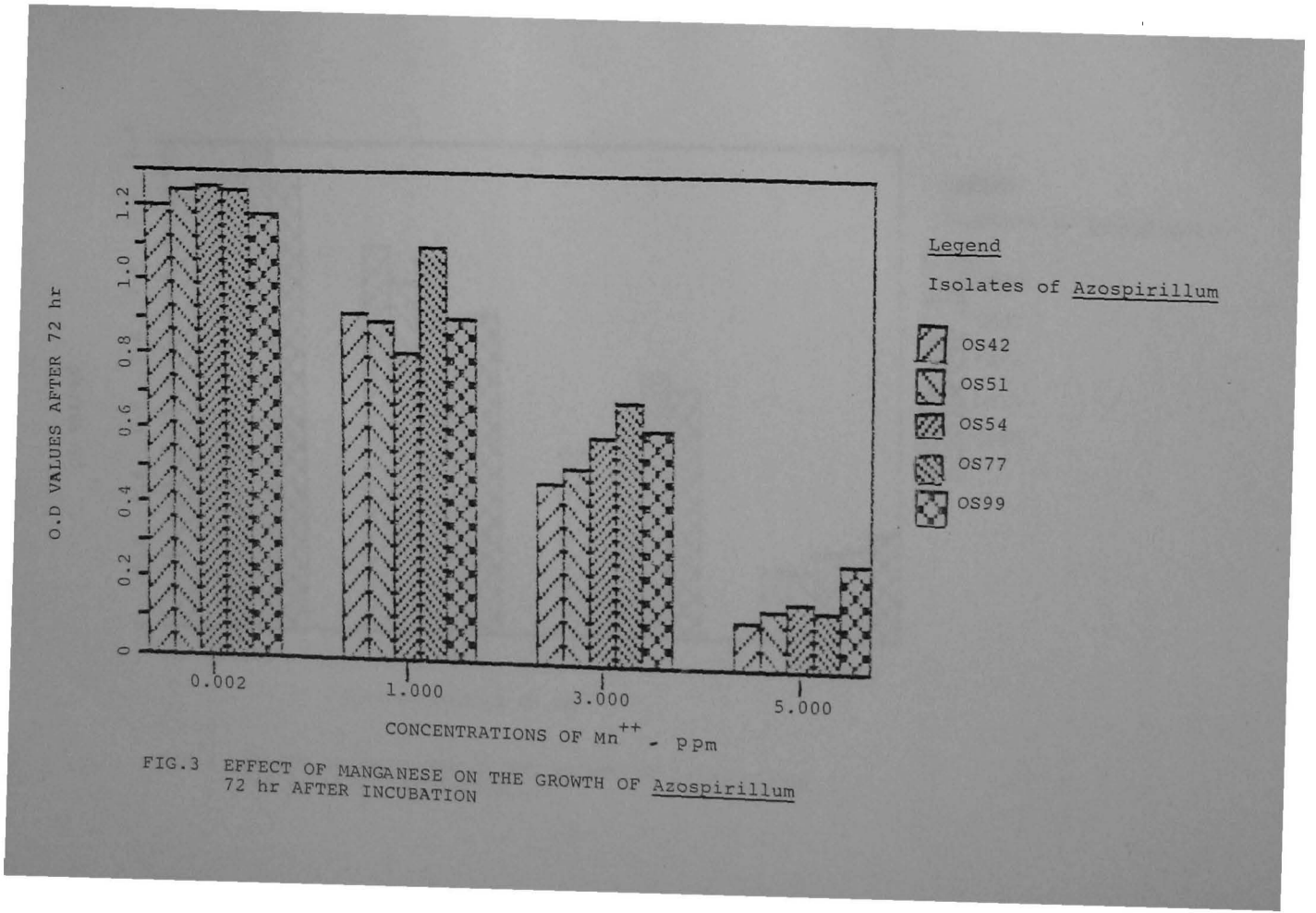


FIG.3 EFFECT OF MANGANESE ON THE GROWTH OF Azospirillum 72 hr AFTER INCUBATION

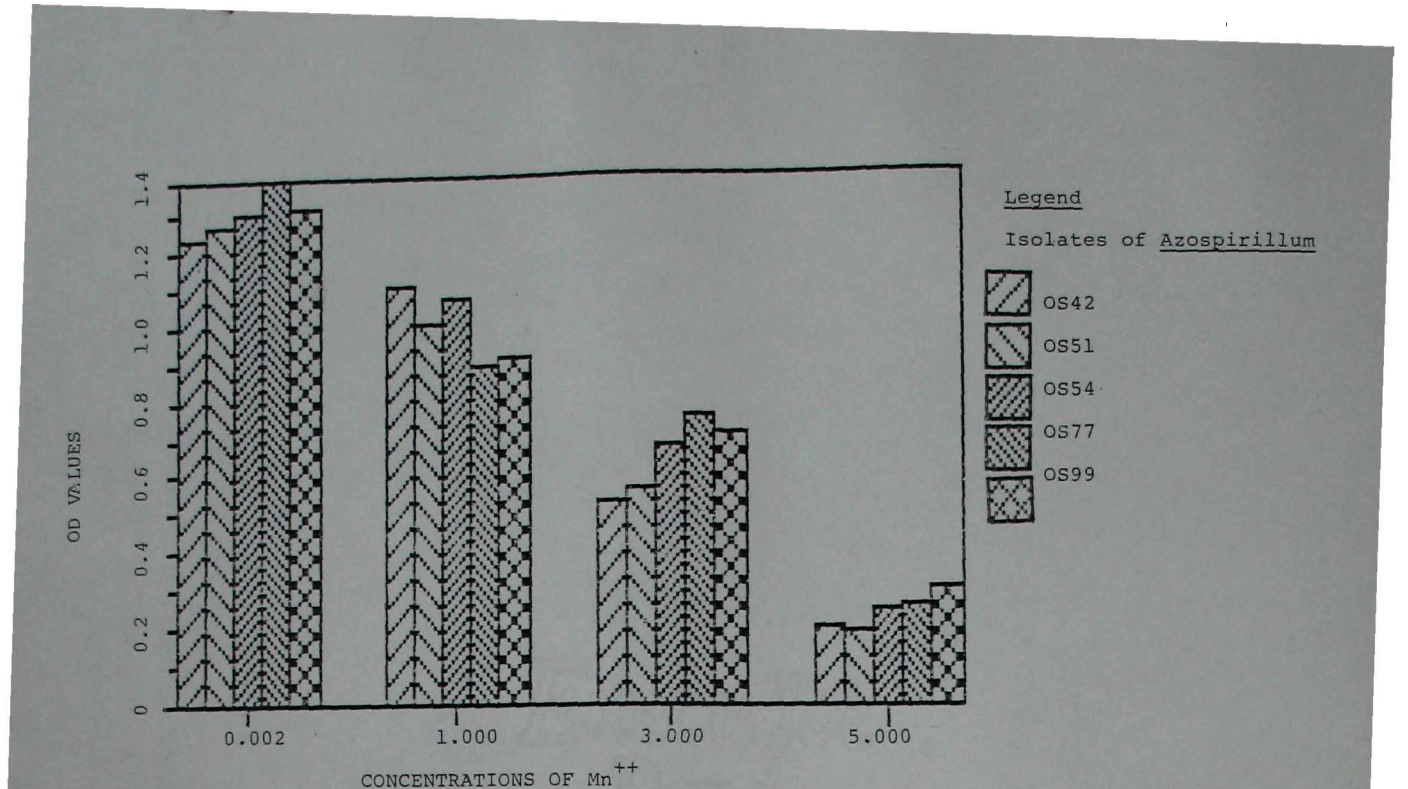


FIG.4 EFFECT OF MANGANESE ON THE GROWTH OF Azospirillum 110 hr AFTER INCUBATION

presence or absence of $MnCl_2$. Those isolates which showed higher tolerance to Mn^{++} were not affected by 1 mM Mn^{++} at the favourable pH. However, when the pH was highly acidic, the toxic effect of Mn^{++} was more and the growth was adversely affected.

4.7 INTRINSIC ANTIBIOTIC RESISTANCE OF *Azospirillum* ISOLATES

The antibiotic resistance of different isolates varied widely (Table 15). About 11% of the total isolates showed resistance upto 50 ppm streptomycin. The percentage of isolates showing resistance up to 40 ppm, 20 ppm and 10 ppm level of the antibiotics were 25.32, 46.83, and 83.54 respectively. Ten per cent of the isolates obtained from Brahmavar soil were resistant to above 50 ppm streptomycin. None of the isolates from Kankanady soil was resistant to 50 ppm level, while 22.2% were resistant at 40 ppm. None of the isolates from Kasargod soil showed resistance at 40 ppm level. However, 25% of the isolates showed resistance at 20 ppm level. About 38% of the isolates obtained from saline soil was resistant to 50 ppm streptomycin. Among the isolates from surface sterilised roots, 16.67% was resistant to 50 ppm streptomycin. Only 5.06% of the total isolates showed resistance to 50 ppm chloramphenicol. About 10% of the soil isolates from Brahmavar, 11% from Kankanady soil and 25% from saline soils were resistant at 50 ppm level.

Table 15. Intrinsic antibiotic resistance of *Azospirillum* isolates

	Streptomycin (ppm)						Chloramphenicol (ppm)					
Isolate	5	10	20	40	50	60	5	10	20	40	50	60
	1	2	3	4	5	6	7	8	9	10	11	12	13
Os 1	+	+	-	-	-	-	+	+	-	-	-	-
Os 2	+	+	+	+	-	-	+	+	-	-	-	-
Os 3	+	+	+	-	-	-	+	+	+	+	-	-
Os 4	+	-	-	-	-	-	+	+	-	-	-	-
Os 5	+	+	+	+	+	-	+	+	+	+	+	-
Os 6	+	+	+	-	-	-	+	+	+	+	-	-
Os 7	+	+	+	+	-	-	+	-	-	-	-	-
Os 8	+	+	-	-	-	-	+	+	-	-	-	-
Os 9	+	+	+	-	-	-	+	+	+	-	-	-
Os 10	+	+	+	+	-	-	+	+	+	+	-	-
Os 11	+	+	-	-	-	-	+	+	-	-	-	-
Os 12	+	+	+	-	-	-	+	+	+	-	-	-
Os 13	+	+	+	+	-	-	+	+	+	+	+	-
Os 14	+	+	-	-	-	-	+	-	-	-	-	-
Os 15	+	-	-	-	-	-	+	-	-	-	-	-
Os 16	+	+	-	-	-	-	+	-	-	-	-	-
Os 17	+	+	-	-	-	-	+	+	-	-	-	-
Os 18	+	-	-	-	-	-	+	+	+	-	-	-
Os 19	+	+	+	+	-	-	+	+	+	+	-	-
Os 20	+	+	+	-	-	-	+	+	-	-	-	-
Os 21	+	+	+	-	-	-	+	+	-	-	-	-
Os 22	+	+	-	-	-	-	+	+	-	-	-	-
Os 23	+	+	-	-	-	-	+	-	-	-	-	-
Os 24	+	-	-	-	-	-	+	+	-	-	-	-
Os 25	+	+	+	+	+	-	+	+	-	-	-	-
Os 26	+	+	-	-	-	-	+	+	+	-	-	-
Os 27	+	+	+	+	-	-	+	+	+	+	-	-
Os 28	+	+	-	-	-	-	+	+	-	-	-	-
Os 29	+	+	+	+	-	-	+	+	+	+	-	-
Os 30	+	+	+	+	+	-	+	+	-	-	-	-
Os 31	+	+	+	-	-	-	+	+	+	-	-	-
Os 32	+	+	+	+	+	-	+	+	+	+	-	-
Os 33	+	+	+	-	-	-	+	+	-	-	-	-
Os 34	+	+	+	-	-	-	+	+	-	-	-	-
Os 41	+	-	-	-	-	-	+	-	-	-	-	-
Os 42	+	+	-	-	-	-	+	+	-	-	-	-
Os 43	+	+	+	+	-	-	+	+	+	-	-	-
Os 44	+	-	-	-	-	-	+	-	-	-	-	-
Os 45	+	+	-	-	-	-	+	+	-	-	-	-
Os 46	+	+	+	-	-	-	+	+	-	-	-	-
Os 47	+	+	+	-	-	-	+	+	-	-	-	-
Os 48	+	+	-	-	-	-	+	+	-	-	-	-
Os 49	+	+	-	-	-	-	+	+	-	-	-	-
Os 50	+	+	+	+	+	-	+	+	-	-	-	-

Table 15. (continued...)

1	2	3	4	5	6	7	8	9	10	11	12	13
Os 51	+	+	-	-	-	-	+	+	+	-	-	-
Os 52	+	+	+	+	-	-	+	+	-	-	-	-
Os 53	+	+	+	-	-	-	+	+	+	-	-	-
Os 54	+	+	-	-	-	-	+	+	+	-	-	-
Os 55	+	+	-	-	-	-	+	+	-	-	-	-
Os 56	+	-	-	-	-	-	+	-	-	-	-	-
Os 71	+	+	+	+	+	-	+	+	+	-	-	-
Os 72	+	+	-	-	-	-	+	+	+	-	-	-
Os 73	+	+	-	-	-	-	+	+	+	-	-	-
Os 74	+	+	-	-	-	-	+	+	+	-	-	-
Os 75	+	+	+	+	+	-	+	+	+	+	+	-
Os 76	+	-	-	-	-	-	+	-	-	-	-	-
Os 77	+	-	-	-	-	-	+	-	-	-	-	-
Os 78	+	+	+	+	+	-	+	+	+	+	+	-
Os 81	+	+	+	-	-	-	+	+	-	-	-	-
Os 82	+	+	-	-	-	-	+	+	-	-	-	-
Os 83	+	-	-	-	-	-	+	-	-	-	-	-
Os 84	+	+	-	-	-	-	+	-	-	-	-	-
Os 85	+	+	-	-	-	-	+	+	-	-	-	-
Os 86	+	+	+	-	-	-	+	+	+	-	-	-
Os 87	+	+	-	-	-	-	+	+	-	-	-	-
Os 88	+	+	-	-	-	-	+	+	+	-	-	-
Os 89	+	+	-	-	-	-	+	-	-	-	-	-
Os 90	+	+	-	-	-	-	+	-	-	-	-	-
Os 91	+	+	+	+	-	-	+	+	-	-	-	-
Os 92	+	-	-	-	-	-	+	-	-	-	-	-
Os 93	+	+	+	+	+	-	+	+	+	+	+	-
Os 94	+	+	+	+	-	-	+	+	-	-	-	-
Os 95	+	+	-	-	-	-	+	-	-	-	-	-
Os 96	+	+	-	-	-	-	+	-	-	-	-	-
Os 97	+	-	-	-	-	-	+	-	-	-	-	-
Os 98	+	-	-	-	-	-	+	-	-	-	-	-
Os 99	+	+	-	-	-	-	+	+	-	-	-	-
Os 101	+	+	-	-	-	-	+	-	-	-	-	-
Os 102	+	+	-	-	-	-	+	+	-	-	-	-
Os 103	+	+	+	-	-	-	+	+	+	-	-	-
Os 104	+	+	+	-	-	-	+	+	-	-	-	-
Os 105	+	+	+	-	-	-	+	+	+	-	-	-
Os 106	+	+	+	+	+	-	+	+	+	-	-	-
Os 108	+	-	-	-	-	-	+	-	-	-	-	-
Os 109	+	+	-	-	-	-	+	-	-	-	-	-
Os 110	+	+	+	+	-	-	+	-	-	-	-	-
Os 111	+	+	+	-	-	-	+	+	-	-	-	-

+ = Presence of growth ; - = Absence of growth

Table 16. Occurrence of salt tolerant Azospirillum in the root environments of saline resistant rice cultivars

Soil types	Cultivar	Concentration of NaCl in the medium											
		0		0.25%		0.5%		1.0%		2.0%		3.0%	
		S	NS	S	NS	S	NS	S	NS	S	NS	S	NS
Mangalore Soil (Non-Saline)	M	+	+	+	+	+	+	-	+	-	+	-	-
	D	+	+	+	+	+	+	-	+	-	+	-	-
	P	+	+	+	+	-	-	-	-	-	-	-	-
	R	+	+	+	+	+	+	-	-	-	-	-	-
Kasargod Soil (Saline)	M	+	+	+	+	+	+	+	+	+	+	-	-
	D	+	+	+	+	+	+	+	+	+	+	-	-
	P	+	+	+	+	-	+	-	+	-	+	-	-
	R	+	+	+	+	+	+	-	+	-	+	-	-
Adyar Soil (Saline)	M	+	+	+	+	+	+	-	+	-	+	-	-
	D	+	+	+	+	+	+	+	+	-	+	-	-
	P	+	+	+	+	+	+	-	+	-	+	-	-
	R	+	+	+	+	+	+	+	+	-	+	-	-

S : Surface sterilised roots NS : Non-Surface sterilised roots

M : Madhu, P : Pushpa, R : Rashi, D : Doddy

4.8 OCCURRENCE OF SALINE TOLERANT *Azospirillum* IN THE ROOT ENVIRONMENTS OF SALINE RESISTANT RICE CULTIVARS

Roots of saline resistant varieties harboured *Azospirillum* tolerant up to 2% NaCl (Table 16). The non-surface-sterilised roots contained the salt tolerant populations frequently. Only roots of Madhu and Doddy grown in saline soils of Kasargod harboured azospirilla tolerant to 2% NaCl. None of the cultivars grown in the selected soils harboured azospirilla tolerant to 3% level of NaCl. The non-surface-sterilized roots of the rice plants (cv.Madhu and Doddy) grown in nonsaline soil of Kankanady had azospirilla tolerant to 2% salt concentration. but they could not be isolated from surface-sterilised roots. Though the non-surface-sterilised roots from all the four cultivars grown in saline soils of Kasargod and Adyar yielded azospirilla tolerant to 2% NaCl, it was not possible to isolate from surface-sterilised roots except from Madhu and Doddy grown in saline soil of Kasargod. In case of Pushpa grown in saline soil of Kasargod it is of interest to note that the non-surface-sterilized roots contained azospirilla tolerant upto 2% NaCl, however it was not possible to obtain the bacteria from surface-sterilised roots.

Table 17. Effect of Azospirillum inoculation at different N levels under field condition on growth of rice cv. Sakthi 30 days after transplantation

Treatment	No. of tillers	Plant height (cm)	Shoot dry weight (g)	Root dry weight (g)
T 1	13.33	54.8	2.19	0.52
T 2	10.33	51.3	1.89	0.45
T 3	11.33	53.8	2.12	0.42
T 4	9.00	49.5	1.59	0.29
T 5	9.67	51.9	1.90	0.38
T 6	8.00	49.0	1.54	0.25
T 7	9.33	50.2	1.74	0.37
T 8	8.00	48.5	1.43	0.22
T 9	7.67	48.3	1.35	0.28
T 10	7.00	45.7	1.33	0.17
C D (0.05)	2.10	4.2	0.51	0.03

T1: Full N (75 kg ha^{-1}) + inoculation T2: Full N
T3: 75 % N + inoculation T4: 75 % N
T5: 50 % N + inoculation T6: 50 % N
T7: 25 % N + inoculation T8: 25 % N
T9: No N + inoculation T10: No N
No inoculation

Table 18. Effect of Azospirillum inoculation at different N levels under field condition on growth and acetylene reducing activity of rice cv. Sakthi 60 days after transplantation

Treatments	Plant height (cm)	Shoot dry weight (g)	Root dry weight (g)	ARA*
T 1	86.3	31.4	1.56	181
T 2	84.5	24.5	0.85	176
T 3	84.0	27.3	0.99	2095
T 4	83.0	22.0	0.77	205
T 5	82.7	26.2	0.88	665
T 6	80.6	21.2	0.78	346
T 7	79.5	21.0	0.84	476
T 8	78.0	19.9	0.73	287
T 9	76.0	20.4	0.82	118
T 10	73.5	19.5	0.66	124
C D (0.05)	2.8	0.20	0.20	119.2

Legend as in table 17

* n moles of ethylene produced g^{-1} dry $root^{-1}$ hr^{-1} .

4.9 RESPONSE OF RICE TO *Azospirillum* INOCULATION UNDER FIELD CONDITION

4.9.1 Root Dry Weight

Azospirillum inoculation increased the root dry weight over uninoculated control. Thirty days after transplanting, all the inoculated treatments showed significantly higher root biomass than their respective non-inoculated control (Table 17). The same trend persisted even 90 days after transplanting. One month after transplanting inoculation together with full N resulted in 0.52 g of root biomass while in the corresponding uninoculated treatment with full N the root biomass was only 0.45 g. With no fertilizer nitrogen, the root biomass was 0.28 g as against 0.17 g in the corresponding inoculated treatment. Two months after transplanting also inoculation resulted in more root biomass (Table 18). Full N + *Azospirillum* treatment had 1.56 g root biomass while the corresponding uninoculated treatment recorded only 0.85 g. The percentage increase over the uninoculated treatment was maximum during the initial periods of growth. On an average, the increase in root biomass due to inoculation was 48.9% at one month stage, 31.7% at second month and it was only 7.83% at the time of harvest.

4.9.2 Shoot Dry Weight

Shoot dry weight was significantly superior due to Azospirillum inoculation at 75% N level over uninoculated treatment at 30 days after transplanting (Table 17). At all other levels of N the effect of inoculation was not significantly different. At 60 days after transplanting, inoculation at 100%, 75% and 50% N levels enhanced the shoot biomass significantly. The increment in biomass production was 28.1%, 24.1% and 24.8%,respectively. At lower doses of fertilizer N, viz. 25% and 0 N, there was no effect due to bacterization.

4.9.3 Plant Height

One month after transplanting, the treatment which received Azospirillum inoculation at 75% N level showed significant difference in plant height over its corresponding uninoculated control (Table 17, Plate 2). Inoculation at any other levels of fertilizer N did not bring about any significant difference. Sixty days after transplanting, inoculated and uninoculated plants were on par with respect to their plant height at all the levels of fertilizer N.

4.9.4 Numbers of Total Tillers and Productive Tillers

At harvest, the total tillers and productive tillers per hill was more in the inoculated treatments than in the uninoculated controls (Table 19). Total number of tillers in

PLATE 2. Effect on Azospirillum inoculation at different N levels under field conditions on growth of rice cv. Sakthi 30 days after transplanting.

Treatments as given under material and methods (Page 57-58)

the treatment which received full N was 9.53, as against 10.2 in the corresponding treatment with inoculation. At other nitrogen levels also the increase was significant varying from 6.3% to 8.5% increase due to inoculation. Azospirillum inoculation also increased the number of productive tillers. Full N + Azospirillum recorded 9.96% increase in productive tillers against the corresponding uninoculated treatment. At 75% N level the number of productive tillers was 9.47 and 8.67 with and without bacterization. Inoculation + 25% N resulted in 7.79% increase over the corresponding uninoculated control. There was no significant difference at 0 N level.

4.9.5 Grain Yield

Azospirillum inoculation resulted in increased grain yield in all the levels of fertilizer nitrogen, varying from 2.72 % to 6.40 % (Table 19, Fig. 5). Full dose of fertilizer N + inoculation gave the highest yield (4.33 t ha^{-1}). Yield obtained in the treatment with full dose of N fertilizer was on par with that of 75 % N + inoculation. Even at lower levels, 25 % N and '0' N, treatments with inoculation were superior to their respective controls.

4.9.6 Straw Yield

Azospirillum inoculation resulted in increased straw yield (2.06 to 10.51%) in all the levels of fertilizer

Table 19. Effect of *Azospirillum* inoculation at different N levels under field condition on growth and yield of rice cv. Sakthi, 90 days after transplantation

Treatments	Plant height (cm)	Total tillers (No.)	Productive tillers (No.)	Grain yield ($T\ ha^{-1}$)	Straw yield ($T\ ha^{-1}$)	Root dry weight (g^{-1} plant)
T 1	94.07	10.20	9.90	4.330	3.189	1.832
T 2	92.47	9.53	9.07	4.195	2.944	1.672
T 3	93.66	10.13	9.47	4.181	3.090	1.747
T 4	92.13	9.53	8.67	3.930	2.831	1.627
T 5	91.86	9.87	9.13	3.981	2.991	1.702
T 6	91.66	9.20	8.49	3.824	2.767	1.619
T 7	89.73	9.67	9.13	3.789	2.806	1.668
T 8	89.53	9.00	8.47	3.681	2.749	1.585
T 9	87.73	7.60	6.67	3.575	2.792	1.348
T 10	87.67	7.10	6.33	3.455	2.602	1.210
C D (0.05)	3.76	0.89	0.75	0.100	0.236	0.068

Legend as in table 17

Table 20. Effect of *Azospirillum* inoculation at different N levels under field conditions on 'N' content and N uptake of straw, grain and root of rice cv. Sakthi

	Grain		Straw		Root		Total N uptake kg ha^{-1}
	Percent N content	N uptake	Percent N content	N uptake	Percent N content	N uptake	
T 1	1.246	53.94	0.866	27.62	0.730	4.45	86.01
T 2	1.221	51.24	0.832	24.50	0.715	3.99	79.73
T 3	1.241	51.88	0.832	25.71	0.706	4.11	81.70
T 4	1.197	47.04	0.810	22.94	0.676	3.67	73.65
T 5	1.231	49.01	0.796	23.80	0.676	3.84	76.65
T 6	1.217	46.53	0.774	21.41	0.664	3.58	71.52
T 7	1.207	45.73	0.749	21.03	0.647	3.60	70.36
T 8	1.148	42.26	0.735	20.20	0.633	3.34	65.80
T 9	1.202	42.97	0.701	19.56	0.628	2.82	65.35
T 10	1.187	41.01	0.696	18.11	0.606	2.44	61.56
CD (0.05)	0.0375	2.45	0.017	2.75	0.0181	0.25	

Legend as in table 17

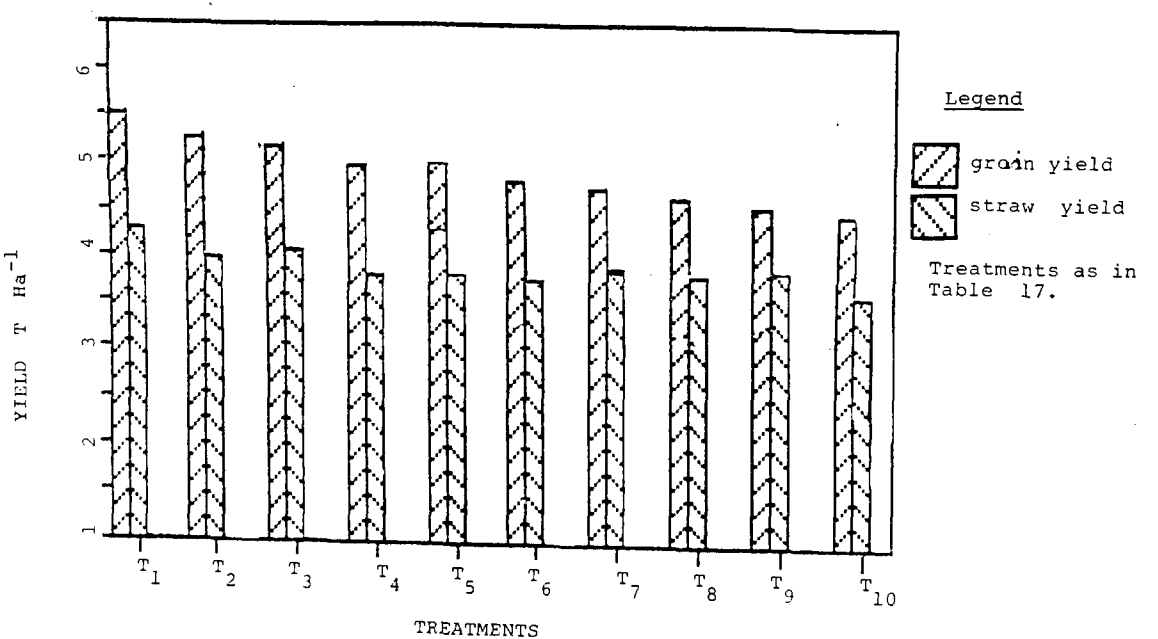


FIG.5. EFFECT OF Azospirillum INOCULATION AT DIFFERENT N LEVELS ON GRAIN AND STRAW YIELD OF RICE CV. SAKTHI.

nitrogen. At 75% N, and 50% N level, inoculation resulted in significant increase in straw yield (6.69% and 10.51% increase respectively). Yield of straw in the treatment 75% N + Azospirillum (3.02 t ha^{-1}) was statistically on par with 100% N alone. At lower levels of fertilizer nitrogen, the effect of inoculation was not significantly different.

4.9.7 N Uptake

Nitrogen uptake by rice increased due to Azospirillum inoculation (Table 20). At 100% N level, nitrogen uptake was 79.33 kg ha^{-1}, while with inoculation it increased to 86.0 kg ha^{-1} (7.88% increase). Nitrogen content of the root, straw and grain was also significantly higher at this level. At 75% N also, the effect of inoculation on the N content of straw, grain and root was significantly higher over its respective control. The N content of the straw in the inoculated treatment did not differ significantly at 50%, 25% and 0 N levels. However, the effect of inoculation on N uptake by grain was significantly more at all nitrogen level except 0 N level; and in roots it was significantly superior at all levels of fertilizer N. Generally bacterization resulted in increased total N uptake at all levels of nitrogen fertilizer, varying from 6.16% to 10.93% with a mean of 7.81%.

Table 21. Effect of *Azospirillum* inoculation at different N levels under field condition on root zone microflora of rice cv. Sakthi after 30 days of transplanting

Treatments	Non-rhizosphere*			Rhizosphere*			Endorhizosphere**		
	Total heterotrophs	*Azospirillum*	N_2 fixers	Total heterotrophs	*Azospirillum*	N_2 fixers	Total heterotrophs	*Azospirillum*	N_2 fixers
	x 10^7	x 10^6	x 10^6	x 10^7	x 10^6	x 10^6	x 10^7	x 10^6	x 10^6
T 1	76.27	4.96	37.26	365.40	91.80	135.60	55.1	0.59	65.1
T 2	22.18	0.60	21.80	132.50	5.40	13.40	26.3	0.27	32.3
T 3	24.05	4.73	26.40	241.80	66.80	106.20	45.7	0.49	52.4
T 4	12.41	0.489	14.80	81.10	4.48	80.30	23.8	0.21	31.6
T 5	22.40	4.10	21.01	145.00	35.06	90.90	36.6	0.28	46.8
T 6	10.50	0.42	12.10	23.50	4.32	29.70	18.4	0.17	27.9
T 7	16.05	4.05	14.10	73.50	26.80	48.30	24.3	0.29	39.2
T 8	13.14	0.31	10.20	16.50	2.32	8.50	12.5	0.11	21.1
T 9	5.41	3.46	8.30	28.80	16.80	22.30	16.2	0.19	25.3
T 10	2.75	0.17	7.10	20.80	2.55	5.20	5.9	0.09	9.5
CD (0.05)	0.73	0.07	2.59	4.26	0.07	2.72	0.92	0.02	0.70

Legend as in table 17

* Population in g^{-1} dry soil
** Population in g^{-1} dry root

4.9.8 Change in the microbial population in the root environment

Initial soil population of Azospirillum in the experimental field was 9.2×10^7 g^{-1} dry weight of soil. A peat based inoculum having a population of 6.00×10^9 g^{-1} was used for inoculation of the seedlings.

After 30 days of transplanting, the MPN counts of Azospirillum was found to be maximum in the nonrhizosphere soil of T_1 (Full N + Azospirillum) (Table 21). Generally inoculation increased the counts of Azospirillum at all levels of nitrogen. On an average, the enhancement was 11.88 times. Even in the control which received no nitrogen fertilizer, the population was 1.73×10^5. Inoculation helped to increase the above population to 3.46×10^6.

Both in the rhizosphere and endorhizosphere, inoculation increased the MPN counts of Azospirillum. In the rhizosphere of the plants which received full dose of N fertilizer, an MPN count of 5.4×10^6 g^{-1} soil was recorded, which was increased by 17 times (91.8×10^6 g^{-1} soil) with inoculation. Inoculation enhanced the Azospirillum population to varying degrees (6.55 to 17.0 times) with an average of 11.63 times. At full dose of N + inoculation, the rhizosphere samples recorded 18.51 times more Azospirillum than the non-rhizosphere samples.

Table 22. Effect of Azozpirillum inoculation at different N levels under field condition on the root zone microflora of rice cv. Sakthi 60 days after transplanting

Treatments	Non-rhizosphere*			Rhizosphere*			Endorhizosphere**		
	Total heterotrophs	Azospirillum	N_2 fixers	Total heterotrophs	Azospirillum	N_2 fixers	Total heterotrophs	Azospirillum	N_2 fixers
	x 10^7	x 10^6	x 10^6	x 10^7	x 10^6	x 10^6	x 10^7	x 10^6	x 10^6
T 1	45.44	26.3	62.4	1003.8	220.0	2750.0	167.3	158.10	381.50
T 2	35.90	22.5	51.8	304.2	42.0	1626.0	112.4	2.89	380.00
T 3	35.13	24.1	46.2	553.6	157.0	1981.0	136.3	73.56	220.40
T 4	34.74	12.4	30.2	226.1	36.0	160.6	88.0	2.00	31.60
T 5	29.06	22.4	32.2	414.2	60.1	1746.0	71.4	50.56	102.40
T 6	20.49	10.5	27.0	100.9	12.8	745.0	51.3	1.26	16.40
T 7	25.42	16.1	20.4	115.7	36.7	933.0	21.6	40.34	43.50
T 8	25.48	13.1	15.3	89.0	6.5	410.0	21.2	0.81	14.20
T 9	20.41	5.4	10.1	112.0	19.9	215.0	20.2	30.12	38.30
T 10	19.42	2.8	8.1	45.8	3.4	110.0	11.4	0.62	12.10
CD (0.05)	0.82	0.9	0.9	7.94	6.27	60.7	4.8	1.59	6.10

Legend as in table 17

* Population in g^{-1} dry soil
** Population in g^{-1} dry root

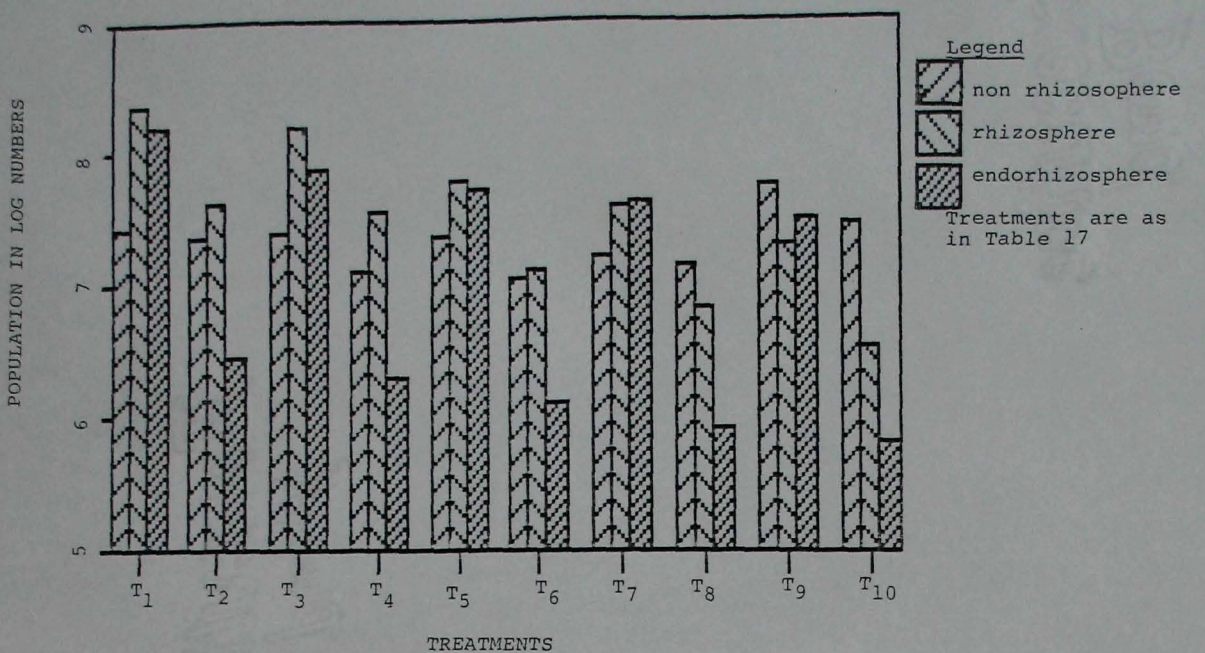


FIG.6 EFFECT OF Asozpirillum INOCULATION AT DIFFERENT N LEVELS UNDER FIELD CONDITIONS ON THE POPULATION OF Azospirillum IN THE ROOT ZONE OF RICE CV. SAKTHI AT 60 DAYS OF TRANSPLANTING

The endorhizosphere also harboured a sizeable number of Azospirillum. The inoculated treatments had higher counts of Azospirillum than the respective uninoculated treatments, which was 1.645 to 2.64 times more, with an average of 2.17. The endorhizosphere population was lesser than the rhizozphere population.

At 60 days after planting, the population of Azospirillum reached the maximum (Table 22 Fig 6). In the treatment with full fertilizer N, the count was 22.5 x 10^5 g^{-1} soil in the non-rhizosphere soil, while it was 26.30 x 10^5 g^{-1} with inoculation. The rhizosphere recorded still higher population; where 100% N with and without inoculation favoured a population build up of 220 x 10^5 g^{-1} soil and 42 x 10^5 g^{-1} soil, respectively.

On comparison of the effects of inoculation on population build up of Azospirillum in non-rhizosphere, rhizosphere and endorhizosphere it can be seen that the effect was more pronounced in the endorhizosphere than the rhizosphere which was again higher than the non-rhizosphere soil. In the non-rhizosphere soil an average of 1.68 fold increase in the MPN counts of Azospirillum was observed in the treatments which received inoculation. The corresponding values for the rhizosphere and endorhizosphere were 5.15 and 46.0. This indicates favourable effect of plant bacteria

interactions in the endorhizosphere and in the rhizosphere and better competitive ability of the inoculated bacteria.

The MPN counts of Azospirillum in the endorhizosphere samples were slightly lower than the rhizosphere. Maximum population of 158.07 x 10^5 was recorded in T_1 (100% N + inoculation) and the lowest count of 0.32 x 10^5 was found in the histosphere of T_{10} (0 N).

The population started declining after 60th day and at the time of harvest T_1 recorded a reduction of 19.60%, 71.74% and 90.95% in the MPN counts of Azospirillum in the non-rhizosphere, rhizosphere and endorhizosphere respectively. All the treatments which received inoculation showed higher population which varied from 1.11-1.72 folds in non-rhizosphere, 2.03-5.01 folds in rhizosphere and 2.04-8.87 folds in histosphere.

Enumeration of the total heterotrophs and diazotrophs revealed that inoculation favoured their build up. The population of heterotrophs and N_2 fixers showed higher counts during 60th day which declined on 90th day.

On 60th day, the population of total bacteria in the non-rhizosphere soil samples of rice plants which received 100% N fertilizer with and without inoculation was 45.44 x 10^7 and 35.90 x 10^7 g^{-1}, respectively (Table 22). The treatment of 100% N + Azospirillum inoculation recorded

Table 23. Effect of *Azospirillum* inoculation at different levels of N under field condition on root zone microflora of rice cv. Sakthi after 90 days of transplanting

Treatment	Non-rhizosphere*			Rhizosphere*			Endorhizosphere**		
	Total bacteria	*Azospirillum*	N_2 fixers	Total bacteria	*Azospirillum*	N_2 fixers	Total bacteria	*Azospirillum*	N_2 fixers
	$\times 10^7$	$\times 10^6$	$\times 10^6$	$\times 10^7$	$\times 10^6$	$\times 10^6$	$\times 10^7$	$\times 10^6$	$\times 10^6$
T 1	30.12	21.12	35.32	128.70	62.17	98.21	55.24	4.81	27.21
T 2	24.20	19.03	29.71	110.30	30.68	81.31	41.33	2.36	20.02
T 3	26.30	18.71	28.81	98.10	57.32	73.54	38.46	3.67	18.18
T 4	21.40	9.63	26.01	75.30	21.23	63.71	29.90	1.05	14.71
T 5	17.61	8.75	25.30	63.60	41.76	59.63	21.70	2.55	13.63
T 6	11.33	7.12	18.21	52.70	16.08	51.83	17.71	0.87	9.71
T 7	10.72	6.34	10.57	48.60	20.71	47.72	15.68	2.14	8.25
T 8	8.71	4.27	8.81	32.30	7.54	42.31	8.31	0.32	6.02
T 9	9.86	3.08	8.03	21.30	10.32	21.12	0.62	1.22	1.07
T 10	7.77	2.68	5.12	18.60	2.06	8.21	0.31	0.14	0.71
CD (0.05)	0.88	2.42	0.54	3.30	1.51	0.71	0.55	0.11	2.44

Legend as in Table 17

* Population in g^{-1} dry soil
** Population in g^{-1} dry root

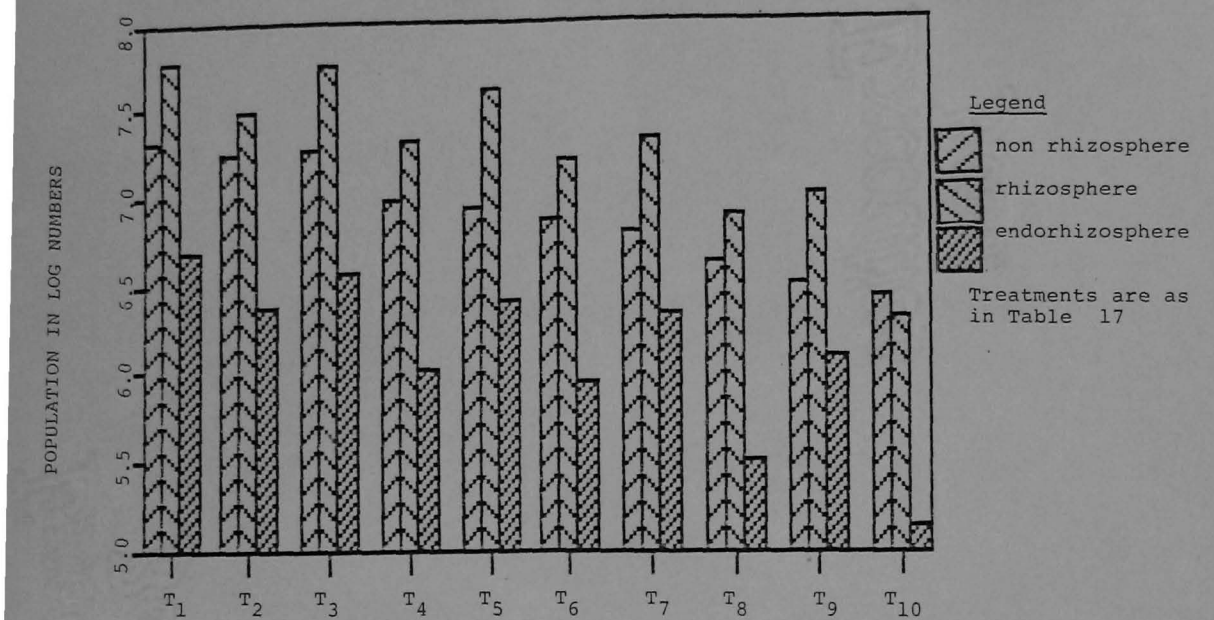


FIG.7 EFFECT OF Azospirillum INOCULATION AT DIFFERENT N LEVELS UNDER FIELD CONDITIONS ON THE POPULATION OF Azospirillum IN THE ROOT ZONE OF RICE CV. SAKTHI AT 90 DAYS OF TRANSPLANTING

maximum bacterial count in the non-rhizosphere soil. All the treatments which received inoculation recorded higher bacterial counts than uninoculated treatments which varied from 1.01 to 1.42 folds increase over the uninoculated treatments with a mean of 1.15. In the rhizosphere maximum population of heterotrophic bacteria was recorded in the treatment with 100% N + inoculation (60.3×10^8). This was 1.98 folds more than the counts obtained in the treatment with 100% N, without inoculation.

In the non-rhizosphere soil the population of diazotrophs on 60th day was maximum in the treatment with 100% N + inoculation (62.4×10^6) which was 1.2 folds more than the population in the treatment without inoculation. Compared to the uninoculated treatments, the corresponding inoculated ones recorded higher counts of N_2 fixing bacteria varying from 1.19 to 1.53 folds increase with a mean of 1.3. The influence was more in the rhizosphere, where 1.23 to 3.34 folds increase was seen in the inoculated treatments over corresponding control. In the histosphere samples also the counts of diazotrophic bacteria were generally more in the treatments which received inoculation. A maximum count of 381.5×10^6 g^{-1} root was observed in 100% N + inoculation which was on par with the corresponding uninoculated treatment. In all other levels of N, significantly higher counts were observed in the inoculated treatments. An

average of 4.08 folds increase was observed in the counts of diazotrophic bacteria due to Azospirillum inoculation.

On 90th day, the rhizosphere soil contained a maximum count of 98.21 x 10^6 g^{-1} soil of nitrogen fixers in the treatment T_1 (100% N + inoculation), while T_2 (100% N alone) recorded 81.31 x 10^6 g^{-1} soil (Table 23). In the non-rhizosphere soil T_1 and T_2 recorded a population of 35.32 x 10^5 g^{-1} soil and 29.71 x 10^5 g^{-1} soil of nitrogen fixers. In the endorhizosphere samples, maximum counts of diazotrophs were obtained in T_1 (27.21 x 10^6 g^{-1} root). This was significantly higher than the counts recorded in T_2 (20.02 x 10^6 g^{-1} root).

4.10 OCCURRENCE OF Chromobacterium

A violet coloured bacterium was found to appear frequently in nutrient agar plates used for studying heterotrophic bacteria associated with rhizosphere of rice roots collected from certain fields of ARS, Kankanady. Their maximum population observed was 20 x 10^6 g^{-1} rhizosphere soil, and in rhizoplane 13 x 10^5 g^{-1} of root dry weight. The pigment production was observed in media supplied with glucose or mannitol or malate, indicating that it is a constitutive character.

The bacterium was identified as Chromobacterium as per Bergey's Manual of Systematic Bacteriology, 1984 (Sneath, 1984) based on the characteristics studied (Table 24).

PLATE 3. Chromobacterium grown in petriplate.

Table 24. Characters used for identifying Chromobacterium

Sl.No.	Characteristics studied	Results
1.	Colony on solid (NA) medium	Violet, smooth, low convex, non-spreading
2.	Gram reaction	negative
3.	Cell shape	Rod, occur in pairs
4.	Motility	Motile
5.	Cell size	0.6 - 0.9x1.5 ~ 3.0 μ
6.	Presence of polyhydroxy butyrate	present
7.	Growth at 37°C	+
8.	Growth at 4°C, 7 days	-
9.	HCN production	+
10.	Arginine hydrolysis	+
11.	Acid but no gas from glucose, frutose, trehalose	+
12.	Gelatin liquefaction	+
13.	Casein hydrolysis	+
14.	Chitin digestion	+
15.	Agar digestion	-
16.	Urease	-
17.	Methylene blue reduction	+
18.	Production of pigment violacin	+
19.	Sole carbon source	
	Citrate	+ slow growth
	L - alanine, L - glutamate--	+
	L - histidine, L - lysine	+
	L - ornithine, L - phenyl alanine--	+

\+ indicates positive reaction
\- indicates negative reaction

The bacterium was Gram-negative rods 0.6 - 0.9 x 1.5 - 3.0 um, often in pairs and contained poly-β-hydroxybutyrate granules as inclusions. Colonies were low convex, violet, smooth and not gelatinous (Plate 3). A violet ring was formed in nutrient broth at the surface with a fragile pellicle. Optimum temperature for growth was 30-35^{o}C, with 10-15^{o}C as minimum and 40^{o}C as maximum. The violet pigment was identified as violacin with the following characteristics.

1. Solubility
   a. Insoluble in carbon disulfide, diethyl ether, chloroform, benzene, toluene and light petroleum.
   b. Slightly soluble in water.
   c. Soluble in ethanol, acetone, amylalcohol, methanol and glycerol. In aniline it gave green solution. Most soluble in pyridine and dimethyl formamide.
2. Chlorine water, bromine water, chromate and permanganate bleached the ethanolic solution of the pigment at once.
3. H_2O_2 did not bleach the pigment unless heated.
4. Treating ethanolic solution with :
   a. Equal volume of 10% KOH, it became green, then turned reddish and then brown in a few minutes.
   b. 50% HNO_2 became yellow orange.
   c. 50% H_2SO_4 gave emerald green colour

Table 25. Population of Chromobacterium observed in root samples of rice collected from certain fields

Field	Rhizoplane * X 10^6 cfu g^{-1} dry root	Rhizosphere ** X 10^6 cfu g^{-1} dry soil
1	0.4	6
2	8	20
3	2	1.3

Table 26. Establishment of seed treated Chromobacterium in the root zone of rice cv. Sakthi

Treatments	Population of Chromobacterium		
	Rhizosphere (X 10^6 cfu g^{-1} dry soil)	Rhizoplane (X 10^6 cfu g^{-1} dry root)	Histosphere
A. Non-surface sterilised seeds treated with bacteria	7.62 x 10^5	16.0 x 10^5	0
B. Surface sterilised seed inoculated with bacteria	4.32 x 10^5	4.11 x 10^5	0

d. Glacial acetic acid gave stable blue colour

e. 2 M HCl gave blue green, turbidity which was stable.

f. H_2O_2 (10% volume) remained violet, stable at 20°C but decolourised at 80°C.

g. 0.1 M $FeCl_3$ became pale yellow.

h. Acetic acid + Zn dust decolourised the pigment.

5. Absorption maximum in ethanolic solution at 579 nm and minimum at 430 nm.

Based on the morphological and biochemical tests, the bacterium was identified as Chromobacterium sp.

Establishment of Chromobacterium in the root environments of 14 day old seedling was studied by inoculating the bacterium to the surface sterilized or non-surface-sterilised seeds of rice cv.Sakthi. The bacterium was found to colonize the rhizosphere as well as the rhizoplane and not the histosphere (Table 26).

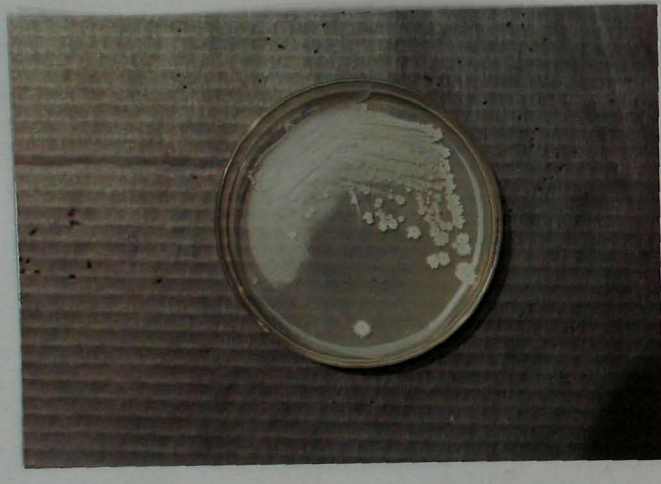
The bacterium was found to grow in hydroxy-apatite medium without showing any zone of solubilization, indicating that it can utilize insoluble forms of P.

4.11 STUDIES ON Bacillus Sp.

While studying the heterotrophic bacteria from the roots of rice plants collected from certain experimental fields at Kankanady, a specific type of bacterial colony was

PLATE 4. Growth of Bacillus sp. in petriplate.

PLATE 5. Growth of Bacillus sp. in semisolid malate medium showing subsurface pellicle.



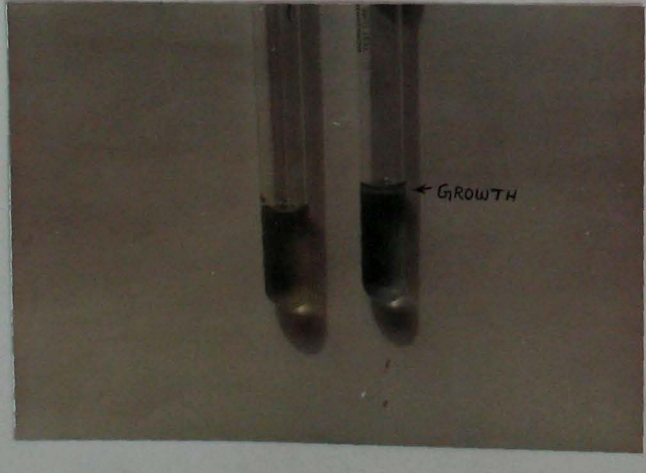

GROWTH

Table 27. Characters used for identifying Bacillus isolated from rice root

Sl.No.	Characteristics	Remarks
1.	Colony characteristics	Centre: highly mucoid, convex and smooth periphery: dry, opaque and irregular margin
2.	Cell shape	Straight rod with rounded ends
3.	Diameter	0.5-0.8 x 1.2-1.9 µm
4.	Production of endospore	+
5.	Motility	+
6.	Gram stain	+
7.	Aerobic growth	+
8.	Catalase	+
9.	Acidification of glucose	+
10.	Formation of indole	-
11.	Growth in 2% NaCl	+
12.	Utilization of	
	lactate	+
	glucose	+
	malate	+
	mannitol	-
	pyruvate	+
	succinate	+

+ indicates positive reaction
- indicates negative reaction

Table 28. Effect of seed inoculation of *Bacillus* sp. on vigour index of rice cv. Sakthi

Sl.No.	Treatments	Root length (cm)	Shoot length (cm)	Vigour Index
1.	Bacterial cell suspension	11.77 (22.8)	6.57 (43.1)	1833.5 (29.3)
2.	Supernatant	11.29 (17.8)	4.95 (8.0)	1624.6 (14.6)
3.	Distilled water	9.58	4.59	1417.5

Data in parenthesis indicate percent increase over control

Table 29. Establishment of seed treated *Bacillus* sp. in the root zone of rice

Sl.No.	Treatments	Rhizosphere ($X 10^6$ cfu g^{-1} dry soil)	Endorhizosphere ($X 10^6$ cfu g^{-1} dry root)
1.	Surface sterilised seeds inoculated with bacteria	28.12	5.95
2.	Non-surface sterilised seeds inoculated with bacteria	16.31	4.34

found frequently on nutrient agar. In such samples their population observed was 165 x 10^6 g^{-1} rhizosphere soil. The histosphere samples also contained counts of 32.5 x 10^6 cfu g^{-1} roots. Interestingly, this bacterium was predominantly present in treatment T_{10} not inoculated with Azospirillum.

The bacterium was isolated in pure culture and its morphological and biochemical characteristics was studied (Table 27).

On nutrient agar medium typical colonies with birds' eye type were formed (Plate 4). The centre of the colony became highly mucoid slightly elevated and smooth, where as the outer periphery remained dry, opaque and with irregular margin.

Cells were rod shaped with rounded ends and straight cells occurred singly and contained endospores. Bacterium was Gram positive and motile.

Based on the characteristics, the organism was identified as Bacillus sp.

On semisolid nitrogen free malate medium they showed subsurface growth (pellicle just below the surface of the medium) (Plate 5) and reduced acetylene. The colour of the medium became blue (alkaline). On hydroxy apatite agar, the

bacterium grew without producing zone of clearing. The bacterium was grown in nitrogen free semisolid malate medium and the acetylene reducing activity was determined. The ARA was found to be 114.8 n moles tube^{-1} hr.$^{-1}$.

Treatment of the rice seeds with the bacterium increased the vigour index. The vigour index was found to be 1833.5 in seeds treated with the Bacillus sp. and 1417 in distilled water control (Table 28).

On seed inoculation, the bacterium established itself in the roots of rice (Table 29). Endorhizosphere of 16 day old seedling (grown from unsterilised seeds and inoculated with Bacillus sp.) contained 4.34 x 10^6 cfu g^{-1} root and its rhizosphere contained 16.31 x 10^6 cfu g^{-1} soil. In case of surface sterilised seeds inoculated with Bacillus sp. establishment was better in both the rhizosphere and the histosphere. The bacterial population was 28.12 x 10^6 g^{-1} soil in the rhizosphere and 5.95 x 10^6 g^{-1} soil in the histosphere.

4.12 EFFECT OF DIFFERENT SOIL MICROORGANISMS ON *Azospirillum in vitro*

P.fluorescence (Strains 14B and 1519), Chromobacterium species and Bacillus sp. were studied for their interaction with five isolates of Azospirillum (Table 30). None of the isolates were inhibited by the bacteria tested. Azospirillum

Table 30. Interaction between few soil microorganisms and *Azospirillum*

Pseudomonas fluorescens	*Azospirillum* isolates tested				
	Os 17	Os 50	Os 89	Os 11	Os 13
1. *P. fluorescens* 140 B	-/-	-/-	-/-	-/-	-/-
P. fluorescens 1519	-/-	-/-	-/-	-/-	-/-
2. *Chromobacterium*	-/-	-/-	-/-	-/-	-/-
3. *Bacillus* sp.	-/-	-/-	-/-	-/-	-/-

-/-: Absence of inhibition of growth in petriplates by different microorganisms on *Azospirillum* or *Azospirillum* on other microorganisms

Table 31. Sources from which *Azospirillum* gains entry into the soil

Sl. No.	Source	MPN counts of *Azospirillum*
1.	Seeds (non-surface sterilised) rice cv. Sakthi	190.0 seed $^{-1}$
	Jaya	152.0 seed $^{-1}$
	Triveni	64.0 seed
2.	Farm yard manure	16.0 x 10^4 g^{-1}
3.	Dissemination through air as indicated by percent of exposed tubes showing growth of *Azospirillum* in different periods	
	1) June	28
	2) July	12
	3) August	16
	4) September	60

also did not inhibit the growth of the test organisms, indicating a neutral relationship. The growth of Azospirillum did not stimulate the other bacteria or vice versa in vitro conditions when grown in petri plates.

4.13 SOURCE FROM WHICH *Azospirillum* GAINS ENTRY INTO THE SOIL

The soil at RARS, Kankanady where the field experiment was laid out had an initial native population of 9.2 x 10^5 g^{-1} soil of Azospirillum. This was found to increase as the crop grew suggesting that the plant root encourages proliferation of Azospirillum. Seeds of the common cultivars of rice contained Azospirillum as an external contaminant - (Table 31). Triveni 64 Nos $seed^{-1}$;Jaya 152.$seed^{-1}$, and Sakthi 190 $seed^{-1}$ Another source was farm yard manure. The farm yard manure which was applied to the field contained 16.0 x 10^4 g^{-1} dry weight. The bacterium was found to be getting disseminated from one field to another through the agency of wind. Sixty per cent of the tubes exposed to air in the field gave positive results during September, while it was only 12% in July.

4.14 SURVIVAL OF *Azospirillum* IN DIFFERENT CARRIERS STORED AT DIFFERENT TEMPERATURES

At $4^{o}C$, the survival of Azospirillum was more in lignite followed by soil + FYM, alginate and perlite (Table 32, Fig 8). The population declined gradually from the date

Table 32. Survival of Azospirillum in different carriers (X 10^8 cfu's g^{-1} dry weight)

A. Stored at 4^oC

Carrier	Period of storage (days)						
	0	15	30	60	90	120	Mean
Lignite	110.0 (100)	81.51 (74.10)	76.0 (69.09)	62.0 (56.36)	52.62 (47.84)	41.0 (37.27)	(56.9)
Perlite	130.4 (100)	73.81 (56.6)	71.2 (54.62)	56.2 (43.08)	32.10 (24.62)	11.01 (8.46)	(37.48)
Alginate	140.1 (100)	65.96 57.86)	61.07 (53.57)	49.67 (43.57)	40.71 (35.71)	26.06 (22.86)	(42.71)
Soil+FYM	70.2 (100)	62.18 (88.57)	51.15 (72.86)	37.61 (53.57)	34.10 (48.57)	22.06 (31.43)	(59.00)
CD (0.05)		(3.98)	(2.37)	(2.60)	(3.55)	(1.14)	(25.22)

Data represent mean of 4 replications
Figures in parenthesis indicate percent population survived compared to the initial population

B. Stored at 10^oC

Carrier	Period of storage (days)						
	0	15	30	60	90	120	Mean
Lignite	120.60	236.60 (196.20)	180.78 (149.90)	150.15 (124.50)	110.47 (91.60)	60.54 (50.2)	(122.47)
Perlite	150.23	163.60 (108.90)	140.16 (93.3)	110.27 (73.4)	63.25 (42.1)	15.32 (10.2)	(65.57)
Alginate	126.80	102.30 (80.68)	82.3 (64.9)	70.12 (55.3)	45.14 (35.6)	28.02 (22.1)	(51.73)
Soil+FYM	74.36	152.8 (205.49)	120.3 (161.8)	103.21 (138.8)	56.51 (76.0)	26.47 (35.6)	(123.53)
CD (0.05)		(25.02)	(13.1)	(12.6)	(6.2)	(3.7)	(68.27)

Data represent mean of 4 replications
Figures in parenthesis indicate percent population survived compared to the initial population

C. Stored at 30°C

Carrier	Period of storage (days)						
	0	15	30	60	90	120	Mean
Lignite	24.11 (100)	62.14 (257.75)	47.09 (195.31)	32.14 (133.29)	12.50 (51.86)	1.26 (5.227)	(128.69)
Perlite	40.30 (100)	55.1 (136.72)	49.10 (121.84)	30.10 (74.69)	10.50 (26.05)	1.30 (3.23)	(72.51)
Alginate	150.10 (100)	282.6 (188.2)	125.71 (83.75)	64.20 (42.77)	1.20 (0.799)	0.705 (0.47)	(63.19)
Soil+FYM	28.31 (100)	64.2 (226.77)	33.10 (116.92)	16.10 (56.87)	7.20 (25.43)	0.60 (2.12)	(85.62)
CD (0.05)		(14.87)	(9.61)	(8.66)	(4.32)	(0.399)	

Data represent mean of 4 replications
Figures in parenthesis indicate percent population to the initial population

D. Stored at 40°C

Carrier	Period of storage (days)						
	0	15	30	60	90	120	Mean
Lignite	110.0	58.3 (53.00)	8.10 (7.36)	0.90 (0.818)	0.07 (0.064)	0.018 (0.016)	(12.25)
Perlite	130.4	46.08 (35.34)	6.22 (4.77)	0.51 (0.39)	0.01 (0.008)	0.012 (0.009)	(8.10)
Alignate	140.1	47.33 (33.78)	11.2 (8.0)	1.10 (0.786)	0.081 (0.058)	0.020 (0.014)	(8.53)
Soil+FYM	70.2	30.28 (43.14)	5.31 (7.57)	0.411 (0.586)	0.02 (0.029)	0.006 (0.008)	(10.27)
CD (0.05)		(2.72)	(0.35)	(0.017)	(0.004)	(0.001)	

Data represent mean of 4 replications
Figures in parenthesis indicate per cent population to the initial population

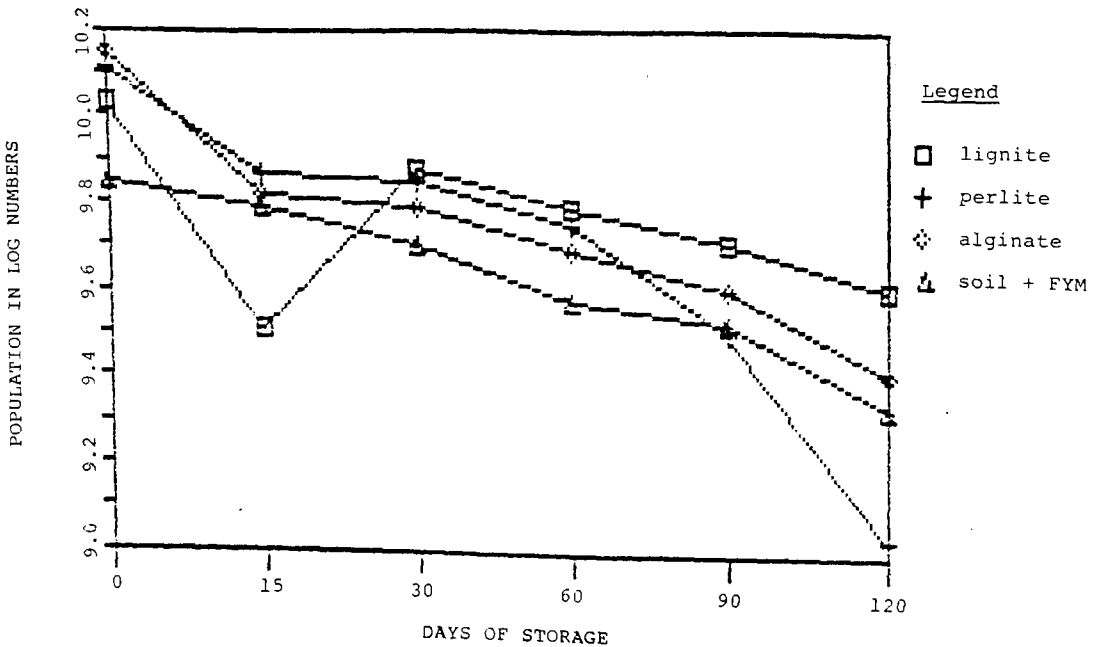


FIG.8 SURVIVAL OF Azospirillum IN DIFFERENT CARRIERS STORED AT 4°C

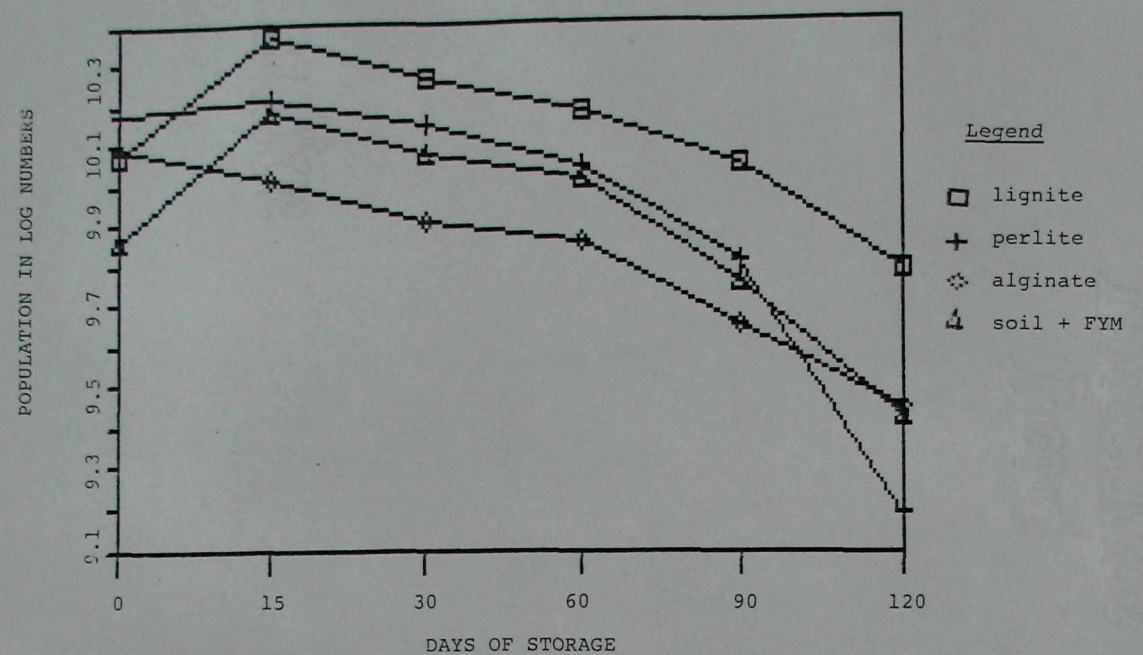


FIG.9 SURVIVAL OF *Azospirillum* IN DIFFERENT CARRIERS STORED AT 10°C

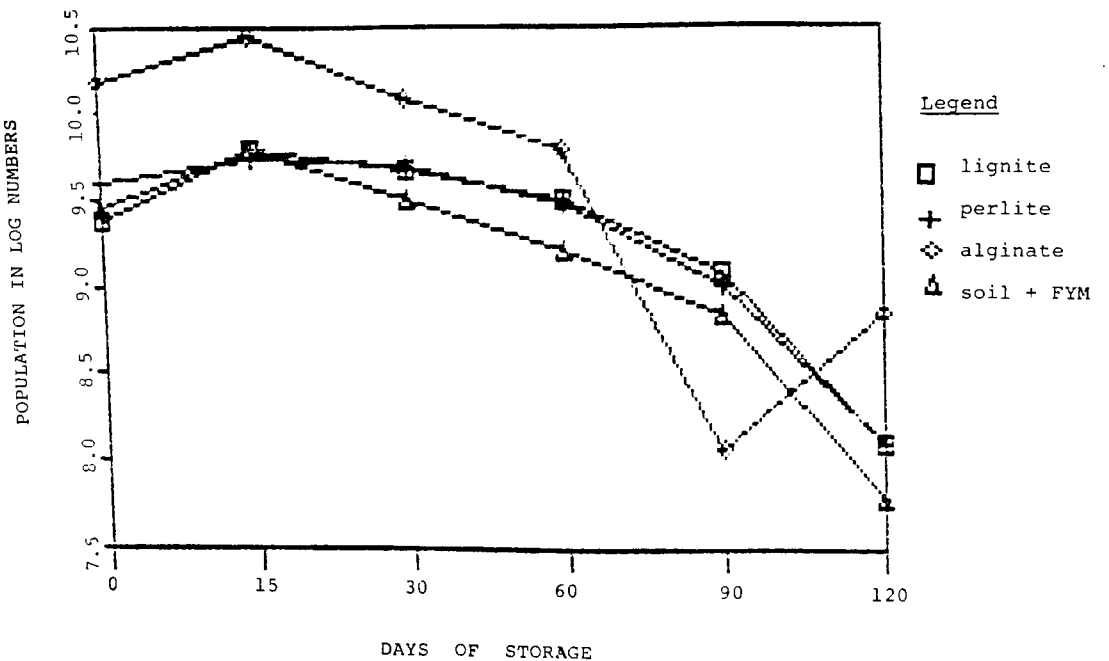


FIG.10 SURVIVAL OF *Azospirillum* IN DIFFERENT CARRIERS STORED AT 30°C

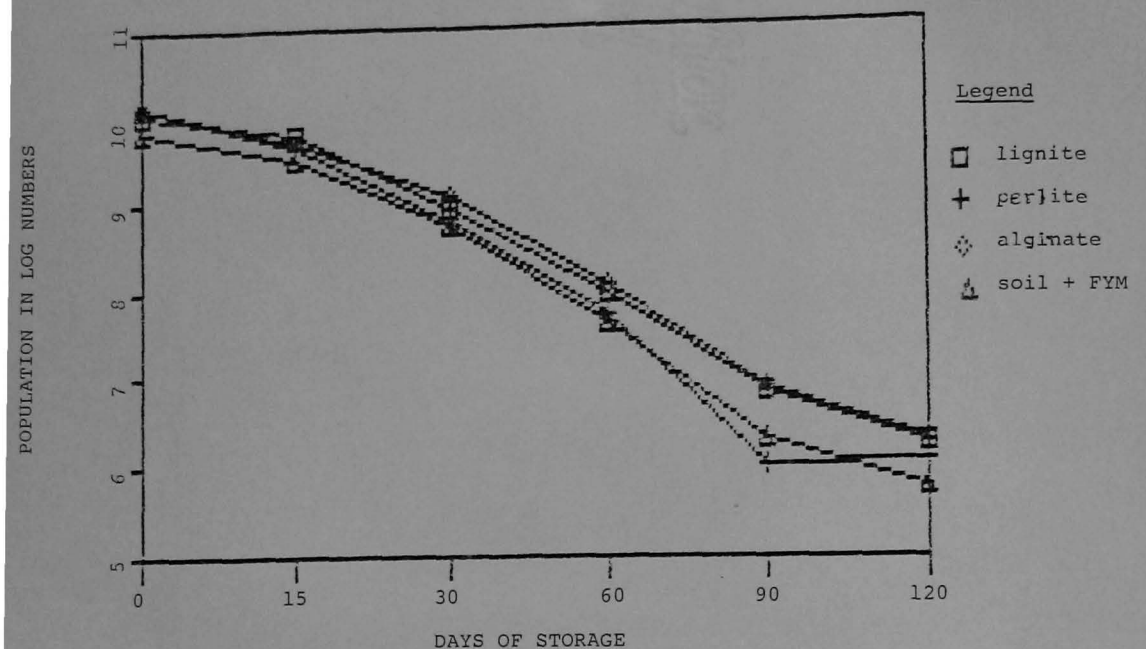


FIG.11 SURVIVAL OF Azospirillum IN DIFFERENT CARRIERS STORED AT 40°C

of storage and at 120 days it had a fairly good number of bacterial cells (41×10^8).

In the carriers stored at $10^{\circ}C$, except in alginate beads, initially there was a slight increase in the population of Azospirillum, which later started declining. (Fig. 9). At 120 days the survival was comparatively better in lignite (60.5% of the original population) followed by alginate beads, soil + FYM and perlite.

The pattern of survival of Azospirillum in lignite, perlite and soil + FYM stored at $30^{\circ}C$ was almost same as above, but the decline was more rapid and at the end of the storage period the bacterial count recorded was only 5.2% of the original count in lignite followed by 3.23% in perlite, 2.12% in soil + FYM and 0.47% in alginate beads (Fig. 10).

The storage temperature of $40^{\circ}C$ drastically reduced the viable cells in the carrier material (Fig. 11). At the end of the storage period the population was only 0.016% of the original population in lignite, followed by alginate beads (0.014%), perlite (0.009%) and soil + FYM (0.008%).

Comparing the effects of different temperatures of storage on the carrier materials, storage at $4^{\circ}C$ was found to retain a higher population, followed by storage at $10^{\circ}C$. Long-term storage at $30^{\circ}C$ or $40^{\circ}C$ was found to lessen the population drastically.

Table 33. Survival of Azospirillum in soil incubated at different temperatures (X 10^8 cfu's g^{-1} dry weight of soil)

Storage temperature	Period of storage (days)						
	0	15	30	60	90	120	Mean
4°C	29.08 (100)	24.60 (84.59)	20.60 (70.84)	13.39 (46.05)	6.50 (22.35)	1.30 (4.47)	(45.66)
10°C	33.12 (100)	25.60 (77.29)	20.20 (60.99)	11.10 (33.51)	2.14 (6.46)	0.43 (1.30)	(35.91)
30°C	34.30 (100)	22.37 (65.22)	20.12 (58.66)	6.38 (18.60)	1.17 (3.41)	0.26 (0.76)	(29.33)
40°C	26.31 (100)	16.48 (62.64)	1.29 (4.90)	0.20 (0.76)	0.06 (0.23)	0.01 (0.04)	(13.71)
CD (0.05)		(7.49)	(34.15)	(2.97)	(2.01)	(0.34)	(42.98)

Data represent mean of 4 replications
Figures in paranthesis indicate per cent population

Table 34. Survival of Azospirillum in soils incubated at different moisture levels and at 30°C (6X 10^8 cfu's g^{-1} dry weight of soil)

	Moisture Level	Period of storage (days)						
		0	15	30	60	90	120	Mean
T 1	Submerged condition	30.60 (100)	110.60 (361.44)	62.10 (202.94)	28.30 (92.48)	12.40 (40.50)	0.76 (2.48)	(139.91)
T 2	Field Capacity	26.40 (100)	31.20 (118.18)	22.50 (85.22)	13.10 (49.62)	11.20 (42.42)	0.66 (2.50)	(59.59)
T 3	50% Field Capacity	34.12 (100)	22.37 (65.56)	17.30 (50.70)	7.10 (20.81)	1.31 (3.84)	0.43 (1.26)	(28.43)
T 4	25% Field Capacity	33.79 (100)	20.20 (59.78)	16.10 (47.65)	6.30 (18.64)	1.08 (3.20)	0.21 (0.62)	(25.98)

Data represent mean of 4 replications
Figures in parenthesis indicate percent population

Lignite was found to be a better carrier material for storage in atmospheric temperature ($30^{o}C$), or at $10^{o}C$ followed by mixture of soil + farm yard manure. At low temperature storage ($4^{o}C$) also, the same trend was observed.

4.15 SURVIVAL OF Azospirillum IN SOIL INCUBATED AT DIFFERENT TEMPERATURE

The soil incubated at $4^{o}C$ always supported a higher bacterial count (Table 33). As the temperature increased, their survival was affected adversely. At the end of the storage period (120 d), the samples kept at $4^{o}C$ contained a population of 1.3×10^{8} cfu g^{-1} of the sample (4.47% of the original count). While it was only 0.01×10^{8} (0.038%) in the sample stored at $40^{o}C$. Soil incubated at $10^{o}C$ and $30^{o}C$ recorded population of 0.43×10^{8} cfu g^{-1} (1.30%) and 0.26×10^{8} cfu g^{-1} (0.76%) respectively.

4.16 SURVIVAL OF Azospirillum IN SOIL AT DIFFERENT MOISTURE LEVELS AND INCUBATED AT $30^{o}C$

The soil moisture level had a profound influence on the survival of Azospirillum (Table 34). At 25% field capacity survival of Azospirillum was very low, and reached a population of 0.21×10^{8} cfu g^{-1} soil (i.e., 0.62% of the original) on 120 days of storage. While at 50% and 100% field capacity it was 0.43×10^{8} (1.26%) and 0.66×10^{8} (2.5%) respectively. However, under flooded condition the

Table 35. Effect of organic manure, oil cakes and nitrogen at two moisture levels on population of *Azospirillum* and heterotropic bacteria

Treatments	Population of *Azospirillum* at different days ($\times 10^5$ g^{-1} dry weight of soil)				Population of total heterotrophic bacteria at different days ($\times 10^6$ g^{-1} dry weight of soil)			
	10d	20d	30d	40d	10d	20d	30d	40d
T 1	20.33	47.67	38.00	22.00	152.00	718.67	218.67	167
T 2	30.33	63.33	42.33	31.33	93.33	460.00	194.00	103
T 3	72.00	150.00	58.33	49.33	411.00	1636.67	810.00	612
T 4	129.67	172.33	148.00	92.00	217.00	1116.67	560.00	410
T 5	43.00	123.33	21.67	18.33	1210.00	2810.00	2140.00	1610
T 6	108.33	143.67	27.67	21.33	1030.00	1936.67	1020.00	1423
T 7	14.33	25.00	16.67	14.00	84.00	182.00	160.00	147
T 8	16.00	31.33	17.33	16.10	61.33	111.00	112.00	102
T 9	-	51.33	50.67	40.00	-	2149.67	1820.00	1120
T 10	-	74.00	63.00	52.00	-	1986.67	1610.00	1010
T 11	-	171.33	160.00	112.33	-	2630.00	1310.00	1007
T 12	-	221.00	183.33	127.33	-	2400.00	1120.00	913
T 13	-	207.33	185.67	121.00	-	3876.67	2870.00	1817
T 14	-	315.00	273.00	183.00	-	1133.33	930.00	712
T 15	-	35.33	19.33	17.30	-	257.00	181.33	162
T 16	-	37.67	25.33	21.10	-	241.00	171.33	153
CD (0.05)	5.63	6.19	5.78	2.93	18.44	25.22	23.48	23.5

Treatment details as given under material and methods (page 69)

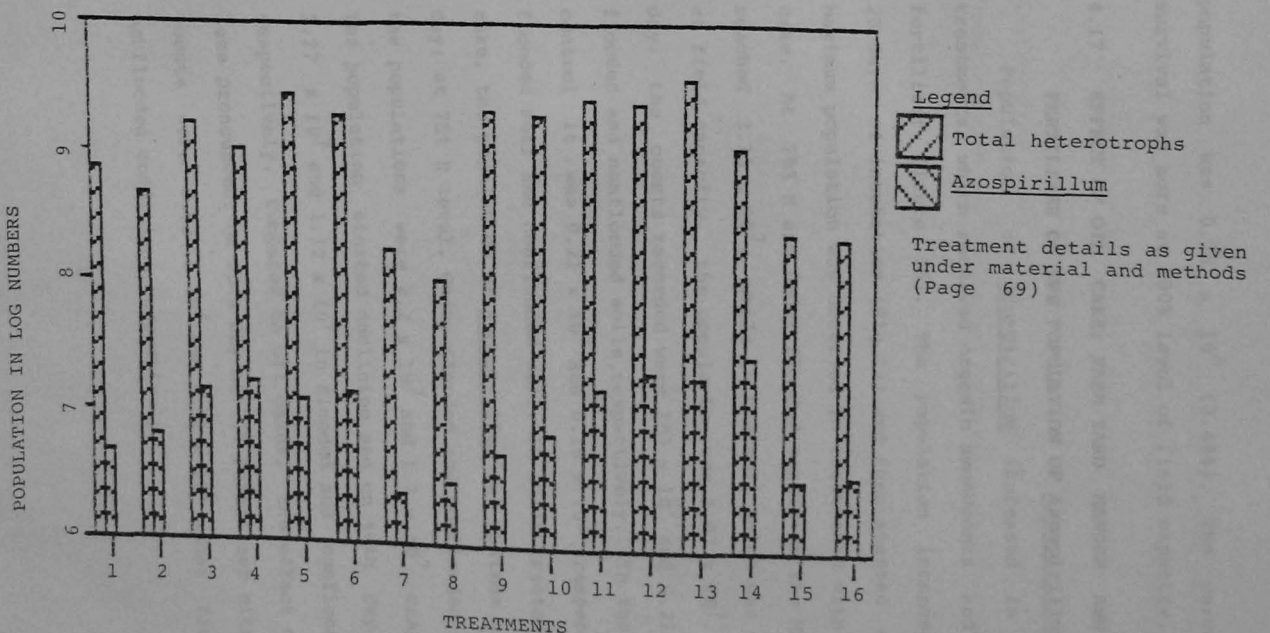


FIG.12 EFFECT OF ORGANIC MATTER ON POPULATION OF TOTAL HETEROTROPHS AND Azospirillum

population was 0.76 x 10^8 (2.48%). The percentage of survival was more at 100% level of field capacity.

4.17 EFFECT OF OIL CAKE; FARM YARD MANURE AND NITROGEN FERTILIZER ON THE POPULATION OF Azospirillum

Population of Azospirillum increased in all the treatments which received organic amendments and nitrogen fertilizer (Table 35). The population increased up to 20 days of incubation (Fig 12) and then started declining. Maximum population was observed in treatments with groundnut cake. At 75% N and under flooded condition the MPN counts reached 3.15 x 10^7; while in the soil which was maintained at field capacity, the population was 2.07 x 10^7. On 40th day, the counts recorded were 183 x 10^7 and 0.21 x 10^7 in flooded and nonflooded soils, respectively. In the unamended control it was 0.21 x 10^7 and 0.16 x 10^7, respectively in flooded soil and nonflooded soil. In soils treated with neem cake, the population of Azospirillum was maximum on the 20th day; at 75% N level. Under flooded and nonflooded treatments the populations were 2.2 x 10^7 and 1.7 x 10^7, respectively. The population started declining and on 40th day, reached 1.27 x 10^7 and 1.12 x 10^7 in flooded and nonflooded soils, respectively. Compared to oil cakes, the effect of FYM was less pronounced. On 20th day at 75% fertilizer nitrogen, the counts were 0.63 x 10^7 and 0.51 x 10^7 under flooded and nonflooded conditions, respectively.

Total bacterial population also reached maximum on 20th day, and it was maximum in nonflooded soil which was amended with ground nut cake and 75% N. Under flooded condition it was only 11.34 x 10^8. On 40th day, the counts of heterotrophs were reduced to 7.12 x 10^8 and 14.23 x 10^8 under flooded and nonflooded conditions, respectively.

DISCUSSION

V. DISCUSSION

Gramineae includes cereals and fodder crops, which are very important in the world agriculture. It was held in view that cereals do not fix nitrogen as there was limited information on the existence of any N_2 fixing organisms associate with. A series of discoveries made since 1974 have clearly established the potentiality of associative nitrogen fixation. The soil bacterium, Azospirillum has been found to be very much associated with the roots of cereals and help in the fixation of nitrogen. The bacterium has been frequently isolated from rice roots (Lakshmi-kumari et al., 1976) and inoculation resulted in enhanced rice yield over uninoculated control and supplemented the nitrogen needs of the crop (Rao et al., 1987). However no systematic study has been carried out with respect to this organism in the acidic soils of coastal Karnataka. Hence, the present investigation was taken up to study the distribution of azospirilla in acidic soils of coastal Karnataka, to isolate and screen them under in vitro and pot conditions, to select an efficient isolate that can be used for inoculating rice crop, and to study the ecological parameters affecting the survival and establishment of the organism.

Native population of Azospirillum in soil samples collected from different locations were enumerated. Seasonal variation of the population and its abundance in different

soil depths were studied. Effect of soil temperature, moisture, and organic matter amendments on the bacteria was determined. Eighty eight isolates of Azospirillum were obtained and characterised. Based on acetylene reduction activity and preliminary screening, an efficient isolate was selected. Using this isolate a field experiment was conducted at ARS, Kankanady, Mangalore to determine the effect of inoculation. Survival of the isolate in different carrier materials was also studied.

5.1 POPULATION DYNAMICS OF Azospirillum IN DIFFERENT SOILS

In the present study, Azospirillum was isolated from acidic soils of rice fields from different parts of coastal Karnataka. Though the pH and salinity of the soil varied, the bacteria recorded fairly high population, clearly establishing that this is a very common soil inhabitant. Studies conducted hitherto by different workers indicate that azospirilla are very common in many parts of the world - tropical, temperate or subtropical in association with different plants like forage grasses, rice, maize, wheat, barley, rye, oats (Dobereiner and De Polli, 1980) and also in different soil types (alluvial, laterite, pokkali and kari) under both upland and lowland conditions (Rao et al., 1978).

In the present experiment also Azospirillum was obtained from upland as well as low land rice fields, and

also from soil samples collected from different soil depths. The populations of Azospirillum in the upland soil of Kankanady was maximum during September (5.07×10^5) and minimum during April (8.53×10^3). The low land and midland had stagnant water during September and in the summer of April, the field was completely dry. Here the population was maximum during December (Lowland 117×10^5, midland 15.6×10^3) and minimum during April (lowland 14.03×10^3, midland 11×10^3). The soil moisture might have profoundly influenced the bacterial population.

The top 10 cm soil recorded higher population during September and December, while the deeper layers of 20 cm and 30 cm recorded higher counts in April. In the upland soil, Azospirillum count in September was 5.15×10^5 and 4.2×10^5 at 20 cm and 30 cm depth, respectively. The population decreased to 1.3×10^5 and 1.8×10^5 in December and recorded a minimum of 13.1×10^3 and 8.4×10^3 in April clearly indicating the influence of moisture stress on the population. As the surface layer gets dried up, the population is drastically reduced. The deeper layers may offer some protection to bacteria by the mulching effect. Similar trend was noticed in lowland and midland also.

The heterotrophic bacteria were maximum in December and minimum in 'April. In the Kankanady upland soil, the populations of heterotrophic bacteria at 10 cm, 20 cm and

30 cm soil were 51.6×10^6, 60×10^6 and 43.0×10^6 in September, 180.3×10^6, 105.3×10^6 and 59.0×10^6 during December and 10.8×10^6, 16.8×10^6 and 4.3×10^6 during April. In low land soils collected from depths of 10 cm, 20 cm and 30 cm the counts of total heterotrophs were 31.3×10^6, 42.6×10^6 and 30.6×10^6 in September, 260×10^6, 318×10^6 and 210×10^6 in December and 15.3×10^6, 42×10^6 and 35.0×10^6 in April.

It can be seen that in the soils of Kankanady, Azospirillum represented only a small percentage of the heterotrophs. It was maximum during September (1.13%) in the upper 10 cm, and drastically reduced to 0.04% in April. The trend was similar in the low land and midland also. In the low land, maximum percentage of Azospirillum was observed in December, representing 0.41% of the heterotrophs. The highest percentage of Azospirillum recorded in the midland was 1.97% at 30 cm depth during September. In the soil samples of Brahmavar, the highest percentage of Azospirillum recorded 0.93% during December at 10 cm depth. The population reached the minimum (0.25%) in April.

Few studies indicate that considerable N_2 fixation occurs in the saline and acid soils (Charyulu and Rao, 1979; Nayak and Rao, 1980). However no systematic information is available on the distribution and activity of Azospirillum in the salt affected soils of coastal Karnataka.

The saline acidic soils of Adyar and Kasargod showed fairly high population of Azospirillum. In the soil sample collected from Adyar, the maximum count of azospirilla (14.4×10^5) was recorded during December in the top 10 cm and the minimum during April (1.1×10^3). At 20 cm and 30 cm depths the bacterial population was found to be 3.2×10^5 and 1.4×10^5, respectively in September. In the soil Azospirillum constituted a maximum of 2.08% of the heterotrophs in September and a minimum of 0.004% in April, indicating the poor ability to survive in the saline soil during temperature and moisture stress. In the saline soils of Kasargod, maximum population of Azospirillum was obtained during September (4.8×10^5) at the top 10 cm layer. A minimum number in the top layer was recorded in April (3.0×10^3). At 20 cm and 30 cm depths, the soil population of Azospirillum was 8.4×10^5 and 3.1×10^5 during September, 3.5×10^5 and 1.2×10^5 during December and 5.0×10^3 and 4.5×10^3 during April. Azospirillum accounted for a maximum of 0.49% in September and 0.01% in April. The present study clearly corroborate the findings of New and Kennedy (1989) that Azospirillum is plentiful in soils above pH 5.0. The present survey conducted on the distribution of Azospirillum in the coastal acidic soils shows its ubiquitous presence and similar pattern of variation with respect to environmental and soil conditions. Sonoria and Prasad (1992) studied the distribution of Azospirillum in Eastern Uttar

Pradesh and found that its occurrence in the soils of three districts was almost alike.

5.2 CHARACTERIZATION OF *Azospirillum* ISOLATES

Azospirillum isolates were obtained from soil/root tissues of rice from all the locations surveyed. Most of the isolates were *A.brasilense* (52.3%). *A.lipoferum* represented only 27.3% of the total 88 isolates. 20.45% of the isolates did not fit into the present described species of *Azospirillum*. Baldani and Dobereiner (1980) postulated host plant specificity in the infection of cereals with *Azospirillum*. They found *A.brasilense* nir^{-1} associated with rice. However, subsequent studies contradicted this proposition. Ladha *et al* (1987) found that 84% of the isolates of *Azospirillum* from rice roots were *A. lipoferum*. Other species of *Azospirillum* viz., *A.halopraeferens* and *A.irakense* also were isolated from rice roots (Balasubramanian and Prabhu, 1989; Khammas *et al*., 1989). The present study is in congruent with the above reports and rule out the possibility of any host specificity with respect to rice roots. *A.lipoferum* and *A.brasilense* obtained from rice soil were 14.78% and 23.86%, respectively (i.e., in the ratio of 1:1.6), while the isolate from surface sterilized roots were in the ratio of 1:2.25. There were strains of azospirilla with atypical characteristics or related organisms which need detailed study to assign proper taxonomic grouping.

5.3 ACETYLENE REDUCTION ACTIVITY IN EXCISED ROOTS OF RICE CULTIVARS AND *Azospirillum* ISOLATES

ARA in the excised roots of twelve cultivars of rice revealed wide variation. The rice cv panama showed a higher activity of 223.4 n moles of acetylene reduced g^{-1} dry root hr^{-1} compared to a lowest activity of 67.4 n moles g^{-1} root hr^{-1} in the cultivar Mahavira. Isolates of *Azospirillum* obtained from the surface sterilized as well as a non-surface-sterilised roots also showed variation with respect to their nitrogen fixing ability. Several investigations suggest variation in the nitrogenase activity associated with different rice varieties (Trolldenier, 1977; Ladha *et al.*, 1985; Rao and Rao, 1985). Ramamurthy *et al.* (1992) reported that acetylene reducing activity of the soil cores and the total diazotrophs varied with the rice cultivar and was maximum in ADT 37. The capacity of a particular rice variety to stimulate N_2 fixation was found to be associated with higher dry matter production and characters linked to photosynthesis (Ladha *et al.*, 1986).

The nitrogenase activity of the isolates recorded considerable variations which ranged from 10.2 to 515.3 n moles of C_2H_4 mg $protein^{-1}$ hr^{-1}. This opened up a way of selecting isolates based on nitrogen fixing ability.

Kavimandan et al. (1981), also observed quite a bit of variations in the nitrogen fixing capacity of their Azospirillum isolates from wheat stems. This admittedly depends upon many factors such as source of carbon, pH of the medium, incubation temperature and dissolved oxygen levels (PO_2) at the site of nitrogen fixation (Day and Dobereiner, 1976; Okon et al., 1976; Nelson and Knowles, 1978). Reports on nitrogen fixing efficiency of Azospirillum strains isolated from different grass crops ranged from as low as 3.4 mg (Quintero and Garzal, 1978) to very high values such as 83.3 mg of nitrogen fixed g^{-1} carbon source consumed (Pedrosa et al., 1980). Acetylene reducing activity of the isolates obtained from soil was less than those obtained from surface sterilised roots. This may be due to the higher distribution of efficient isolates among those obtained from surface sterilized roots. A plausible explanation is that rice roots may be preferentially colonized by more efficient nitrogen fixers. It has been reported that the ecto and endorhizosphere are colonized by different strains of Azospirillum (Baldani et al., 1986). Low population of efficient isolates in soil and their access to the plant roots may be a limiting factor for associative N_2 fixation. This clearly explains the need for isolation, screening and inoculation to assure the build up of efficient strains in the root zone of rice.

5.4 EFFECT OF SEED INOCULATION WITH *Azospirillum* ON VIGOUR INDEX OF RICE SEEDLINGS

Seed inoculation resulted in significant increase in vigour of rice. Most of the isolates increased vigour and the effect of inoculation was more pronounced on root development. This is in agreement with the observation made by Dhanapal *et al*. (1978). They observed that inoculation of seeds with *S.lipoferum* increased the vigour index of sorghum, maize, sunflower, bhendi, cotton and rice. Maize seedlings soaked in *Azospirillum* culture showed pronounced root and shoot elongation after five days of growth (Nair, 1981). Kalandre and Konde (1992) recorded a significant stimulatory effect of *Azospirillum* on seed germination and plumule and radicle lengths of wheat seedlings.

Treatment with 16 isolates failed to enhance the vigour by causing deformation of roots and decreasing the root length. All of them enhanced the shoot length. There are reports of root deformations caused by growth factors produced in excess by the plant in response to root colonization by *Azospirillum* (Horemans *et al*., 1986). Bashan (1986) found decrease in root surface area due to increased population (10^8 cfu ml^{-1}) of *Azospirillum*.

Rinaudo et al. (1981) tested Azospirillum spp. isolated from rice and found differences in plant response to different isolates. The isolates which were selected based on their influence on vigour index of paddy seedling also performed well in the preliminary screening in pots. Inoculation with OS 99 resulted in 78.5% increase in seedling biomass over control. Gunasekaran et al. (1992), reported increased shoot and root length in rice plant due to inoculation of Azopirillum

5.5 EFFECT OF HEAVY METALS ON GROWTH OF Azospirillum

Soil acidity factors (high Al and Mn and low pH and Ca) are known to adversely affect the survival, growth and nitrogen fixation by microorganisms (Munns, 1977). Selection of strains having high tolerance to heavy metals and lower pH can be expected to perform better under field conditions. The present study clearly showed varying degrees of tolerance among different isolates. Nearly 42% of the isolates were tolerant to 100 mM concentration of $AlCl_3$. The percentage of isolates tolerant to 5 mM, 10 mM and 50 mM levels of MnCl2 were 45%, 16%, and 6.5%, respectively. About 29% of the total isolates were tolerant to 400 mM FeEDTA, while 6.45% showed growth even at 1000 mM level. Azospirillum strains on growth at higher concentrations of $MnCl_2$ were found to produce brown pigment. It was reported that under unfavourable conditions, A.brasilense form cysts

(Lamm and Neira, 1981) accompanied by the production and excretion of brown black melanin like granular pigments (Sadasivan and Neyra, 1987).

All of the isolates tested showed poor growth beyond 3.0 mM concentration of $MnCl_2$. However isolate OS 99 was less sensitive to Mn^{++} toxicity. Increasing level of $AlCl_3$ in the medium adversely affected the growth of Azospirillum. Among different isolates tested, OS 99 showed better growth at 20 ppm level of $AlCl_3$.

pH below 6.0 slowed the growth of Azospirillum under in vitro conditions both in the presence or absence of $MnCl_2$. Those isolates which showed higher tolerance to MN^{++} was not affected by 1 mM Mn^{++} at the favourable pH. However when the pH was highly acidic, the toxic effect of Mn^{++} was more, and the growth was adversely affected. The pH ranges for optimum growth of A.amazonense, A.lipoferum and A.brasilense strains from a variety of habitats were found to be 5.7-6.5, 5.7-6.8 and 6.0-7.3 , respectively (Baldani et al., 1986). Rai (1986) studied the manganese resistance of native Azospirillum, and found declining pattern of growth and N_2 fixation at and over 55 μg Mn ml^{-1} and ultimately stopped at 275 μg ml^{-1}. Some of the mutants of A.brasilense showed cross resistance to Al^{+++} 660 μg Mn^{-1} ml^{-1} + 2.5 μg Al ml^{-1}) and showed higher nitrogenase activity.

5.6 INTRINSIC ANTIBIOTIC RESISTANCE OF *Azospirillum*

Azospirilla live in a highly competitive soil environment. Resistance of higher level of antibiotics may help to survive better. This character can be used as a marker for strain differentiation and ecological studies. Isolates of *Azospirillum* varied widely in their ability to tolerate different levels of antibiotics. About 25% of the total isolates were resistant to 40 ppm level of streptomycin, while only 5% were resistant to 50 ppm chloramphenicol. Various authors have reported *Azospirillum* strains possessing different levels of antibiotic sensitivity. Baldani and Dobereiner (1980) observed that most *Azospirillum* strains were killed at 5 ppm streptomycin concentration and use of 20 ppm streptomycin helped to isolate higher percentage of resistance strains. Contradictory to this, Franche and Elmerich (1981) found that all the wild type strains of *A.lipoferum* and *A.brasilense* are sensitive to streptomycin, rifampicin, nalidixic acid, cephaloridin and cefalotin. Singh and Wensel (1982) reported that all the strains of *A.brasilense* and *A.lipoferum* are resistant to trimethoprim except *A.lipoferum* A23 which was highly sensitive. Magalhaes *et al.* (1983) observed that all the *A.amazonense* strains are resistant to penicillin, but relatively tolerant to chloramphenicol and erythromycin. On perusal of the present results and the earlier works it is evident that *Azospirillum* strains have

different tolerance level to various antibiotics. Existence of ecological races of soil microorganisms was upheld by Mishustin and Yemtsev (1982). They reported that one and the same microbial species, inhabiting the soils of different climatic zones differ in some physiological features.

5.7 EFFECT OF *Azospirillum* INOCULATION AT GRADED LEVELS OF NITROGEN ON PLANT GROWTH AND YIELD

Studies on the response of rice cv. Sakthi to *Azospirillum* inoculation under field condition showed a positive response to inoculation. Inoculation increased the root dry weight over uninoculated control. Thirty day after transplanting all the inoculated treatments showed significantly higher root biomass than their respective non-inoculated control. The same trend continued up to 90 days after transplanting. The present study has reaffirmed that inoculated plants produce higher root biomass than the control. Dewan and Subba Rao (1979) reported that root biomass of rice seedlings increased due to inoculation with *A.brasilense*. One of the earliest and best described events in *Azospirillum* root colonization is the impact on root development. (Michielis *et al.*, 1989). The inoculated mature plants showed a more developed root system in all cases (Patriquin *et al.*, 1983; Jain and Patriquin, 1984). Tien *et al.* (1979) proposed that these changes in root morphology are caused by plant growth factors produced by *Azospirillum*.

They demonstrated the production of auxins, gibberellins and cytokinins in pure cultures of A.brasilense. They were able to mimic the inoculation effect on root morphology by supplying a mixture of these growth factors to the rooting medium. Recently Kundu and Sangwan (1992) established the role of phytohormones produced by Azospirillum. Mutants with low production of phytohormones and with high nitrogenase activity showed reduced ability in promoting root systems, while those strains overproducing indole compounds increased plant biomass and N content.

Production of total tillers and productive tillers per hill was more in the inoculated treatments than in the uninoculated control at all levels of fertilizer nitrogen. There was no significant difference at 0 N level. Watanabe and Lin (1984) reported early tillering and reproductive growth and increased filling rate of the grain due to inoculation with A.lipoferum.

Inoculation with Azospirillum resulted in increased grain yield at all the levels of fertilizer N. Full dose of fertilizer N + inoculation gave maximum yield (4.33 t ha^{-1}) which was superior to all other treatments. Yield obtained with full dose of fertilizer N was on par with that of 75% N+ inoculation. Even at lower levels, 25% N and 0 N, treatments with inoculation were superior to their

respective controls. Inoculation also resulted in increased straw yield in all the levels of fertilizer N. At 75% N and 50% N level, the increase was significant. At lower levels of fertilizer N, the effect of inoculation was not significantly different. The results are incongruent with the observation made by Rao et al. (1983) who observed a statistically positive yield response to inoculation with Azospirillum sp. at different levels of N fertility over three consecutive seasons. Though Azospirillum inoculation failed to give consistent positive response, many reports have appeared indicating the bacterium as a potential microbial inoculant for rice (Subba Rao et al., 1979; Nayak et al., 1986; Kannaiyan et al., 1983; Govindaswamy et al., 1992; Sharma et al., 1992).

Acetylene reducing activity of the excised roots of the inoculated plants was higher than uninoculated plants. ARA was maximum at 75% N + inoculation. Earlier studies indicate that rice-Azospirillum association is capable of fixing N_2 more efficiently at low levels of fertilizer N under field conditions (Nayak and Rao, 1977; Rao et al., 1983).

The effects of inoculation are generally believed to be due to increased N_2 fixation and nitrogen input. A temporary increase in nitrogenase activity occurred due to inoculation, but this increase became insignificant as the rice plant grew older (Rao and Rao, 1983; Watanabe and Lin,

1984). These results indicate that the beneficial effects of inoculation may not be related solely to N_2 fixation. There are reports showing lower and similar rates of N_2 fixation in the rhizosphere of inoculated compared to uninoculated control plants (Rao and Rao, 1983; Nayak et al., 1986).

The present study revealed that nitrogen uptake by rice increased due to bacterial inoculation. The increased grain and straw yields obtained may be due to the increased N uptake by rice plants as a result of Azospirillum inoculation. This is in conformity with the earlier findings (Watanabe and Lin, 1984; Nayak et al., 1986; Kumar and Balasubramanian, 1989).

The rhizocoenoses formed between Azospirillum and rice plants involve an intimate mutual interaction between both partners. This takes place in the rhizosphere or within the root tissue and depends on many complex interactions between the rice plant, microorganisms and the ecosystem. The colonization is the result of a selective enrichment of the microorganism best adapted to the ecological niche formed by the root environment. The present study showed that inoculation of rice with Azospirillum helped in the establishment of the association. MPN counts of Azospirillum increased in the rhizosphere due to inoculation. The population was maximum on 60th day after transplanting and then started declining. The endorhizosphere also harboured

sizeable population of the bacterium. However it was lesser than rhizosphere population. This may be the reason why Scott et al. (1978) observed a sharp decrease of Azospirillum population by sterilization with chloramine T, as surface sterilization ostensibly reduces the organism in the rhizoplane.

Some Azospirillum strains penetrate the roots of their host and become established in high numbers in the intercellular spaces between the epidermis and the cortex or in the outer cortical layers (Okon et al., 1977; Patriquin and Dobereiner, 1978) and even in the vascular system (Patriquin et al., 1983). A successful invasive strain is specifically adapted to survival and/or proliferation in the inner root environment. Okon et al (1988) concluded that under appropriate agronomic conditions, roots of grasses colonized at early stages of growth with an optimal number of Azospirillum have positive effects on crop yield.

5.8 SOURCE FROM WHICH Azospirillum GAINS ENTRY INTO THE SOIL

Azospirillum is considered to be a soil bacterium. Under the influence of plants, its population increases. The study revealed that Azospirillum can gain entry into the soil or root environments via different means. Soil is the main source of the bacterium. Non-surface sterilized seeds and farm yard manure also contributed the initial source of

Azospirillum. Hegazi et al. (1979) showed the presence of Azospirillum in phyllosphere of maize and sugarcane and on seeds of maize. Wind dissemination of the organism as revealed by its growth in tubes exposed to air current reveals a transport mechanism other than the root to root movement (Bashan and Levanony, 1987a) or limited self motility in the soil (Bashan, 1986). The present finding supports the earlier work of Bashan (1991) that in controlled environments, plants inoculated with A.brasilense caused contamination of non-inoculated plants via. air transmission. They detected the air transportation of Azospirillum up to 6 m from the inoculated source. The air borne Azospirillum cells probably become airborne from the surface soil, which are known to contain indigenous Azospirillum.

5.9 OCCURRENCE OF NITROGEN FIXING Bacillus sp.

A bacterium that frequently encountered in plates while enumerating heterotrophic bacteria from rice roots was identified as Bacillus sp. The bacterium was closely associated with rice roots. In semisolid malate medium it was able to grow and fix atmospheric nitrogen. It was able to grow in hydroxy apatite medium without producing clearing zone, indicating its ability to utilise water insoluble forms of P. On seed inoculation the bacterium got established on root surface and endorhizosphere. Seed

bacterization increased vigour index of seedlings. The organisms of the Bacillus type which include B.polymyxa, B.macerans and B.circulans fix N_2 under anaerobic conditions (Kapulnik, 1991). Seldin et al. (1984) isolated an efficient N_2 fixing bacterium, B.azotofixans from Brazilian soils and grass roots. The abundance of this bacterium in the uninoculated control plants and very low population in the rice plants inoculated with Azospirillum under the field conditions of Kankanady may be due to the better competitive ability of the inoculated Azospirillum to occupy the energy rich habitats of the rhizosphere and endorhizosphere. However, the higher numbers of Bacillus sp. in the uninoculated control may be due to its better competitive ability than other microorganisms.

5.10 OCCURRENCE OF Chromobacterium sp.

A violet pigmented bacterium was found to occur frequently in the rhizosphere soil samples collected from rice grown in the Agricultural Research Station, Kankanady. The bacterium was characterised and identified as Chromobacterium. The genus was reported to occur in soil and water (Sneath, 1960). Its role in the rhizosphere is not known. The bacterium was found to grow in hydroxy apatite medium without showing any zone solubilisation, indicating that it can utilise insoluble forms of P. On seed inoculation, the bacterium established in the root

environments, rhizosphere and rhizoplane, but was absent in the endorhizosphere suggesting its inability to enter the intact roots. The high population under natural field conditions (20 x 10^6 g^{-1} rhizosphere soil) rules out the possibility of this bacterium being a chance contaminant through rainwater or other means.

While studying the population of Azospirillum from fresh roots, and soil fluorescent pseudomonads were found very frequently in the enrichment medium. It formed pellicle in the NFb medium (Baldani and Dobereiner, 1980) and gave a positive ARA.

5.11 INTERACTION OF Azospirillum WITH CERTAIN SOIL BACTERIA

The three bacterial isolates viz., Bacillus sp., chromobacterium sp. and Pseudomonas fluorescens were studied for their interaction with Azospirillum in vitro. None of the three bacteria either enhanced or inhibited Azospirillum. Similarly Azospirillum did not influence the growth of the 3 bacteria tested. There is a report that Azospirillum grew better and fixed more nitrogen when cocultured with cellulose degrading bacteria (Halsall and Gibson, 1985).

5.12 EFFECT OF MOISTURE AND TEMPERATURE ON THE SURVIVAL OF Azospirillum IN THE SOIL

Soil moisture is one of the most important factors deciding the population of soil microorganisms. Azospirillum

being a microaerophilic organism (Dobereiner et al., 1976) can survive better in soils containing high moisture status. In the present study, submerged condition and field capacity supported the survival of Azospirillum better than 50% and 25% of field capacity. Soil moisture having a profound influence on ^{15}N incorporation in rice soil was reported by Charyulu and Rao (1979).

The present study clearly brought out the influence of temperature on the survival of Azospirillum in storage. Of the different storage temperature (4°C, 10°C, 30°C and 40°C) studied, 4°C was the best followed by 10°C. However the bacterium was able to withstand higher temperatures to a certain level. Even at 40°C, their population survived in the soil, may be by arresting metabolic activities. It is well established the vegetative cells of Azospirillum have the capacity to form resistant structures (cysts) which may help the organism to survive under unfavourable conditions (Lamm and Neyra, 1981).

5.13 SURVIVAL OF Azospirillum IN DIFFERENT CARRIERS

The development and testing of carrier materials suitable for large scale use and distribution is essential for extending the use of microbial inoculants. Survival of the inoculant in the carrier material is a prerequisite of a good carrier material. In the present study among the carrier materials tested, Azospirillum survived better in

lignite and soil +FYM. Its survival was low in perlite. Storage at $10^{o}C$ and $4^{o}C$ retained more bacteria. Encapsulation in alginate pellets supported the survival of Azospirillum to a lesser extent compared to lignite and soil + farm yard manure. About 22% of the bacteria survived in alginate beads stored at $10^{o}C$ for 120 days. But it was only 0.47% when the storage temperature was $30^{o}C$. Though alginate pellets cannot be used as commercial carrier material for use of Azospirillum in cereal crops, it can be thought of as a suitable one for plantation crops due to its advantages over others. Moreover more than one organism can be immobilized in this system and hence their synergistic effects can be exploited easily. Tilak et al (1979) found that soil and farm yard manure mixed 1:1 and sterilized for 3 hr daily on 3 consecutive days was a suitable carrier for Azospirillum.

5.14 EFFECT OF SOIL MANAGEMENT PRACTICES ON Azospirillum

The contribution of N_2 fixation to the fertility of rice fields can be enhanced by selected improved management practices such as organic amendments (Rao, 1978; Watanabe and App, 1979), suitable water and fertilizer management (Ladha et al., 1986) etc. The present study indicate that groundnut cake, neemseed cake and farm yard manure increase the population of Azospirillum, groundnut cake being the best. The influence of farmyard manure on Azospirillum

population was less pronounced compared to oil cakes. When soil was amended with organic matter, the population of Azospirillum increased up to 20 days of incubation and then started declining. Depending on the nature of organic material added and the soil conditions, the behaviour of different flora will vary. On addition of succulent plant materials to the soil, bacterial numbers increased rapidly and by seventh day began to decline (Alexander, 1977). Chandrasekhara Rao (1979) also reported increase in the population of Azotobacter in the rhizosphere of citrus as well as soils treated with neem cake, groundnut cake or castor cake.

In the present study, the counts of heterotrophic bacteria also increased by the addition of farm yard manure and oil cakes including neem cake. This is in contrast with the observation of Mishra et al. (1972) that application of neem cake to soil reduced the number of soil bacteria and actinomycetes. However the effect of neem cake on different kinds of microorganisms may vary. Mishra et al. (1975) demonstrated that neem cake application did not affect the counts of nitrate forming organisms. Negi et al., (1980) recorded higher population of Azospirillum in the rhizosphere of barley grown in soils amended with organic matter, such as city compost and rice straw compost.

The present study indicates that flooded conditions favour the build up of Azospirillum compared to non-flooded condition. On 40 days of incubation, neem cake and groundnut cake treated soil had 1.35 folds and 1.47 folds more population of Azospirillum than non-flooded soil. This upholds the view of Jena and Rao (1988) that Azospirillum population increased under flooded condition. Nayak and Rao (1981) observed several fold increase in nitrogenase activity following a shift from non-flooded to flooded condition.

To develop a suitable technology for augmenting nitrogen fixation and enhancing rice yield, efficient microbial inoculants for specific agroclimatic zones have to be developed. In this direction the present study brings out clearly the following.

1. Azospirillum is a very common soil and root inhabitant in acidic soils or saline acidic soils of coastal Karnataka.
2. Azospirillum isolates vary in nitrogen fixing efficiency and in enhancing crop growth.
3. High soil moisture and addition of organic matter increase the population of Azospirillum.
4. Higher concentration of heavy metals like aluminium and manganese adversely affect growth of the organism.

5. Screening trials and field experiments revealed that A.brasilense OS 99 is very effective for inoculating rice in acidic soils of coastal Karnataka.
6. Chromobacterium sp. and a nitrogen fixing Bacillus sp. were frequently observed in association with rice roots.
7. Lignite can be used as a carrier material for large scale field application of Azospirillum.
8. Immobilization of the bacterium in alginate matrix can be an alternative proposition compared to the usual carrier materials used for inoculation.

SUMMARY

VI. SUMMARY

The present investigation was undertaken to study the biocoenosis of Azospirillum in relation to rice (Oryza sativa L.) grown in the acidic soils of coastal Karnataka. Population dynamics of the bacterium in different soils, its interaction with biotic and abiotic factors along with their utilitarian value for rice as a biofertilizer were studied. The results are summarized hereunder.

1. Different locations representing non-saline acidic soils (Brahmavar, Kankanady and Kasargod) and saline acidic soils (Adyar and Kasargod) were selected and soil samples collected from depths of 0-10 cm; 11-20 cm and 21-30 cm. Population of azospirilla was enumerated in different seasons following serial dilution and MPN method. Azospirilla were frequent in all the soils studied irrespective of their soil characteristics and depth. In the upland soils of Kankanady, Azospirillum population was maximum during September and minimum during April. In the lowland and midland the population was maximum in December and minimum during April. The total heterotrophic bacteria was maximum during December and minimum during April. In Brahmavar soil, azospirilla were maximum during December and minimum during April. Among the different depths maximum population was found at top 10 cm in

December and at 10-20 cm in April. In the soils of Kankanady, Azospirillum represented only a small percentage of the total heterotrophic bacteria. It was maximum in September in the top 10 cm, which drastically reduced to 0.04% in April. Saline acidic soils of Kasargod and Adyar also showed fairly good population of Azospirillum. In the soil sample from Adyar, maximum count of Azospirillum was recorded during December in the top 10 cm soil.

2. By enrichment culture technique, Azospirillum isolates were obtained from soil and root tissues of different cultivars of rice. Their cultural and biochemical characteristics indicated that 55.7% was A.brasilense and 22.3% were A.lipoferum. Some of the isolates were showing properties which were not typical of the recognised species. No host plant specificity with respect to a particular species of Azospirillum and rice plant was detected.

3. Isolates of Azospirillum varied in their ability to fix atmospheric nitrogen. Isolates of Azospirillum obtained from surface sterilized roots were more efficient nitrogen fixers. Acetylene reducing activity of the excised roots of twelve cultivars of rice showed wide variation. The rice cv. Panama showed the highest activity and IR36 the least activity.

4. Among the 31 isolates of Azospirillum studied for their tolerance to heavy metals 80.6% showed tolerance to 10 mM concentration of $AlCl_3$, while 41.9% showed tolerance to 100 mM concentration. Only 6.5% of the isolates were tolerant to 50 mM concentration of manganese. Most of the isolates showed fairly high levels of tolerance to FeEDTA. About 29% of the isolates were tolerant to 400 mM FeEDTA while 6.5% showed tolerance even at 1000 mM level. Presence of 3.0 mM $MnCl_2$ in the medium adversely affected the growth of the bacteria. However, the isolate OS 99 was less sensitive to Mn^{++} toxicity.

5. Seed treatment of rice with Azospirillum sp. resulted in increased vigour index. The isolate OS 77 recorded maximum vigour index with 26.3% increase over distilled water control. Twenty one isolates showed more than 10% increase in vigour index over control.

6. In a preliminary screening trial in pots, 25 isolates of Azospirillum were screened to select an efficient isolate for inoculating rice. Maximum plant length and biomass was observed in plants inoculated with the isolate A.brasilense OS99. Hence the isolate was selected for field inoculation.

7. Intrinsic antibiotic resistance of different isolates was studied. Only 11% of the isolates showed resistance

up to 50 ppm of streptomycin, while 5.0% showed resistance up to 50 ppm chloramphenicol.

8. Response of rice to Azospirillum inoculation was studied under field conditions at ARS, Kankanady. Inoculation enhanced root and shoot growth, number of productive tillers, grain yield and straw yield. Full dose of fertilizer N + inoculation gave maximum grain yield (4.3 t ha^{-1}). At 75% N and 50% N level inoculation resulted in significant increase in straw yield. At 25% and 0 N, the effect of inoculation on straw yield was not significantly different from their respective uninoculated controls. N uptake of rice increased from 7.9% to 10.6% with a mean of 8.7% due to inoculation.

9. Inoculation resulted in increased colonization of Azospirillum in the rhizosphere and endorhizosphere. At recommended level of N + inoculation, the rhizosphere samples had 18.5 times more Azospirillum compared to the non-rhizosphere samples. The endorhizosphere also harboured a sizeable number of Azospirillum. Maximum Azospirillum population was recorded in plants after 60 days of transplanting. Population of Azospirillum in the rhizosphere increased up to 60 days after transplanting but declined later. Inoculation also resulted in an increased build up of heterotrophic bacteria and total diazotrophs.

10. A violet pigmented bacterium was found to appear frequently in the rhizosphere soil samples collected from rice fields of ARS, Kankanady. The bacterium was identified as Chromobacterium sp. On seed inoculation, the bacterium colonized the rhizosphere and rhizoplane, but not the endorhizosphere.

11. Azospirillum was isolated not only from soil but also from the non-surface sterilised seeds and farm yard manure. The experiment conducted suggests that Azospirillum can be disseminated by air.

12. Among the different carrier materials tested, lignite and soil + farm yard manure were found to be more suitable as carrier material for Azospirillum. The bacterium was successfully immobilised in alginate pellets. Its survival was lower in alginate pellets compared to lignite and soil + farm yard manure. In the carrier based inoculum Azospirillum survived for a longer period when stored at $4^{o}C$ and $10^{o}C$.

13. Soil moisture had a profound influence on the survival of Azospirillum. Low moisture content (25% field capacity) drastically reduced its population while its survival was better at 100% field capacity and flooded condition.

14. Soil amendments like farmyard manure or oil cakes increased the build of Azospirillum. Maximum population was observed in treatment with groundnut cake at 75% N,

under flooded condition. The population reached a maximum on 20th day and then started declining.

Considering the data presented, it may be concluded that Azospirillum is a major diazotroph in the root environment of rice and inoculation with the selected isolate can enhance plant growth and yield.

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VII. REFERENCES

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* Original not seen

APPENDICES

APPENDIX - 1

CHEMICAL ANALYSIS OF SOIL AT ARS, KANKANADY

Organic Carbon (%)	:	1.05
pH	:	5.8
Electrical conductivity (mmhos/cm at 25°C)	:	0.37
Aluminium (meq/100g)	:	0.98
Iron (ppm)	:	86.67
Manganese (ppm)	:	4.13

APPENDIX II

a. Composition of N free semi solid malate medium (NFb medium) (Baldani and Dobereiner, 1980)

Malic acid	:	5.0 g
Dipotassium hydrogen orthophosphate	:	0.5 g
Magnesium sulphate	:	0.2 g
Sodium chloride	:	0.1 g
Calcium chloride	:	20.0 mg
FeEDTA (1.64% w/v; aqueous)	:	4.0 ml
Trace element solution	:	2.0 ml
Alcoholic solution of bromothymolblue (5%)	:	2 ml
Vitamin solution	:	1.0 ml
Potassium hydroxide	:	4.0 g
Agar	:	1.75 g
Distilled water	:	1000 ml

pH adjusted to 6.8 with 1.0 N NaOH

i. Trace element solution

Sodium molybdate	:	200 mg
Manganese sulphate	:	235 mg
Boric acid	:	280 mg
Copper sulphate	:	8 mg
Zinc sulphate	:	24 mg
Distilled water	:	200 ml

ii. Vitamin solution

Biotin	:	10 mg
Pyridoxin	:	20 mg
Distilled water	:	100 ml

b. Potato infusion agar for non selective growth and purity checks (BMS) : (Baldani and Dobereiner, 1980).

Potatoes	:	200 g
Malic acid	:	2.5 g
Potassium hydroxide	:	2.0 g
Sucrose	:	2.5 g
Vitamin solution	:	1.0 ml
Agar	:	20.0 g
Distilled water	:	1000 ml

c. Congored medium for purity checks (Rodriguez-Caceres, 1982).

K_2HPO_4	:	0.5g
$MgSO_4.7H_2O$	:	0.2g
NaCl	:	0.1g
Yeast extract	:	0.5g
$FeCl_3.6H_2O$	:	0.015g
Malic acid	:	5.0g
KOH	:	4.8g
Agar	:	20.0g
Congored (1:400 aqueous solution)	:	15.0 ml
Distilled water	:	1000 ml