DEVELOPMENT OF INOCULANT CULTURES OF *Azospirillum* FOR RICE (*Oryza sativa L.*) IN KUTTANAD

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THESIS

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DECLARATION

I here by declare that this thesis entitled ***"DEVELOPMENT OF INOCULANT CULTURES OF Azospirillum FOR RICE (Oryza sativa.L.) IN KUTTANAD"*** *is a bonafide record of reserch work done by me during the course of research and that the thesis has not previously formed the basis for the award to me any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.*

Trivandrum

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CERTIFICATE

Certified that this thesis entitled "Development of inoculant cultures of Azospirillum for rice in Kuttanad" is a record of research work done independently by Sri.Sasi Kumar.S under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Affectionately Dedicated
to
My Father

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ABBREVIATIONS

DAT	-	Days After Transplanting
IAA	-	Indole Acetic Acid
IBA	-	Indole Butyric Acid
MSL	-	Mean sea Level
NFb	-	Nitrogen free bromothymol blue medium

CHAPTER I

INTRODUCTION

Nitrogen is the key nutrient in plant growth. It goes into the constitution of plant proteins, chlorophyll, nucleic acids and many other plant substances. Hence, the deficiency of nitrogen will result in poor crop yields. Nitrogen is supplied to the crop plants mostly in the form of nitrogenous fertilizers. The cost of nitrogen fertilizers has increased many fold during the recent years and the trend still persists. The indiscriminate use of chemical fertilizers to boost the crop yield has created an alarming situation, where in the soil properties including biological factors are adversely affected. The shortage of high protein plant food together with the alarming concern about the fast depleting energy sources has stimulated much interest in biological nitrogen fixation.

The biological nitrogen fixation involves reduction of atmospheric nitrogen in the presence of enzyme nitrogenase to ammonia which is the key intermediate in the synthesis of many other nitrogen compounds in the cell. Atmospheric nitrogen can be used directly by a number of free living and symbiotic prokaryotes. Nitrogen fixation in cereals and grasses in association with certain bacteria remains one of the major research challenges in agriculture and bacteriology. Most of

these plants support nitrogen fixation in their roots and this contributes directly to the productivity of Graminae. The important non-symbiotic nitrogen fixing bacteria are Azospirillum, Azotobacter, Beijerinckia and Derxia.The nitrogen fixing capacity of associative nitrogen fixing bacterium Azospirillum is unquestioned and has considerable potential in agriculture.

Kuttanad (Fig.1) is one of the major rice growing tracts of Kerala. This low lying (-1.5-2.5 MSL) region is unique in its soil characteristics like high organic matter content, high acidity and is prone to frequent salt water inundation from the sea. Azospirillum is an important biofertilizer used in rice cultivation as its contribution to nitrogen economy in rice fields is significant. It fixes about 20-25 kg/ha nitrogen and produces certain plant growth promoting substances which are known to improve root development and seedling growth of rice plants. The generally used Azospirillum biofertilizer fail to establish and perform in the Kuttanad region due to the failure of introduced cultures to withstand the unique soil conditions prevailing in the region. Moreover, there is no record of earlier work to develop inoculant culture of Azospirillum for the highly acidic soils of Kuttanad. The present investigation is intended to develop an efficient native inoculant culture of Azospirillum for the acidic tracts of Kuttanad.

CHAPTER II

REVIEW OF LITERATURE

The importance of soil fertility, especially soil nitrogen, for crop production was emphasized as early as 1834 by experiments of French agriculturist, J.B. Bounsingault. This was followed by the classical work of Justus Von Leibig, a German chemist, on soil nutrients. He prepared a balance sheet of plant nutrients and soil nutrients. J.B. Boussingault also proposed that legumes possess the capacity to fix atmospheric nitrogen in the soil. The discovery of legume root nodules in 1886 by Hellreigel in Germany and the Rhizobium as a causative agent in the fixation of atmospheric nitrogen in nodules by Dutch scientist Beijerinck (1888) are considered as milestones in the field of biological nitrogen fixation. Since then innumerable reports on nitrogen fixation by various microorganisms and related aspects have appeared in the literature.

Azospirillum is an associative nitrogen fixing bacteria which grows in association with various Graminae and other host plants. They occur freely in rhizosphere also. But fixes nitrogen only under microaerophilic condition. It was Beijerinck(1922) who described a vibroid soil bacteria that seemed to fix atmospheric nitrogen. It was initially called as

Azotobacter spirillum and later he renamed it as Spirillum lipoferum (1925). He found that mixed cultures containing this bacteria showed increased nitrogen content whereas cultures lacking this did not. But pure cultures failed to grow in nitrogen deficient media. No studies on this organism appeared in the literature until 1976, except a few scattered ones, when Dobereiner and Day(1976) isolated a similar organism from roots of tropical grasses. They demonstrated that these organisms fixed nitrogen under microaerophilic conditions. Tarrand et al.(1978) revised the nomenclature of Spirillum and designated it as Azospirillum.

Azospirillum biocoenosis/rhizocoenosis

Azospirillum is known to colonise on a wide range of host plants. The variety of carbon compounds present in the root exudates serve as energy source and make the rhizosphere a suitable habitat for high microbial activity. Dobereiner and Depolli (1980), Ruschel and Vose(1981) at the "International Workshop on Associative Nitrogen Fixation", Brazil suggested "Diazotrophic Biocoenosis" as a general term for the associative nitrogen fixation and "Diazotrophic Rhizocoenosis" as a more appropriate term for nitrogen fixation in, on or close to the roots.

Dobereiner and Day (1976) were the persons first to report "Azospirillum rhizocoenosis". Even though Azospirillum can colonise on a wide variety of plants, there exists some sort of host plant specificity with maize, wheat and rice (Baldani and Dobereiner, 1979). They found that, of the different isolates studied, 58% of the maize isolates were A.lipoferum and 100% of wheat isolates and 96% of isolates from rice were A.brazilense. Besides their reports also showed that Azospirillum isolates associated with wheat and rice were A.brasilense nir^-. Azospirillum occurs widely in the roots of cereal crops, grasses and tuber crops. It is also found free living in rhizosphere soils (Kreig and Dobereiner, 1984). Azospirillum occurs in the stem of many plants. It was found in the stem of wheat (Kavimandan et al., 1978), rice (Watanabe and Borraquio, 1981) and in the roots and stem of rice and grasses (Khan and Hossain, 1990).

Many scientists have studied the mode of invasion and colonisation of Azospirillum in host plants. Azospirillum colonises roots both externally and internally. On external colonization they form aggregates and enhance mucigel production and a large number of them can be found embedded in it. Further they found that Azospirillum invades seedling via middle lamella of older root tissue and transparent areas around the invading

of older root tissue and transparent areas around the invading cells. This is achieved by active hydrolysis of plant cell walls by proteolytic enzymes. The production of enzyme was also noticed in culture media (Umali and Gracia, 1978).

The colonisation by the bacteria on roots vary according to the host plant. In wheat most of the bacterial population was internal whereas in pearl millet the majority was concentrated on root surface (Mathews et al., 1983). In Kallar grass there was high surface colonisation and internal colonisation was found to be nil (Reinhold et al., 1986). Reports show that external colonisation was mostly on root hairs and root elongation regions (Bashan, 1986, Okon and Kapulnik, 1986). In cereals colonisation was mainly on root surface rather than on root hairs(Bashan,1986). Live and dead roots were found colonised by Azospirillum. (Bashan and Levamony, 1988 a).

Occurrence and ecological distribution

Spirillum lipoferum is a very common soil and root inhabitant in tropics, subtropics and temperate regions of the world (Dobereiner and Day, 1976). Initially its occurrence was noticed and isolation made only from tropical grasses. But later they found its nitrogenase activity with roots of temperate zone

plants such as Festuca rubra. Silva de and Dobereiner (1977) observed that eventhough European soils under grass roots harboured Azospirillum, their number was considerably less when compared to those in tropics. The nitrogen fixation by Indian isolates of Azospirillum was maximum at temperature ranging from 30 to 35^{o}C (Subba Rao et al., 1980).Water stress has been found to have a considerable effect on the rhizosphere population and percentage root infection of Azospirillum (Banwari Lal and Rao, 1990).

The presence of Azospirillum in different parts of the world characterised by different ecological conditions has been reported by many scientists. Its occurrence in the soil samples and roots has been reported in Germany, Australia, Belgium, U.S.A., South Africa and South America(Schroder, 1932; Dobereiner, 1967; Sloger and Owens, 1976; Quniterio and Garzia, 21977; Tyler et al., 1979).

Rao et al., (1978) noted the presence ofAzospirillum in different soil types like alluvial, laterite, pokkali and kari. Charyulu and Rao (1980) found that Azospirillum occurs in acid soils with pH as low as 3.2 and in alkaline and saline soils with pH of 8 to 8.8 (Purushothaman and Oblisami, 1985). The occurrence of Azospirillum with roots of a number of tropical forage grasses, (Balandreau, 1975; Dobereiner and Day, 1976;

Neves et al.,1976). Rice, (Kumari et al., 1976; Rao et al., 1978) wheat, (Neyra and Dobereiner, 1977), aquatic plants,Sylvester Bradley, 1977) temperate grasses, (Glatzle and Martin, 1981) plantation crops (Govindan and Purushothaman, 1985) and cotton and soyabeans (Bashan, 1991) has been reported..pl60

Morphology and physiology

Azospirillum is a gram negative, aerobic bacteria which has a typical curved rod shape, motile with a polar flagellum and contains polybeta hydroxy butyrate (PBH) granules and the DNA base composition is of 69-71 mole percent G+C.(Tarrandel al., 1987).

Sugars in general are poor carbon sutrates for A. lipoferum. It grows moderately on galactose or acetate but poorly on glucose or citrate. Optimal growth and nitrogenase activities are observed when S.lipoferum is grown on malate, succinate, lactate and pyruvate (Day and Dobereiner, 1976; Dobereiner and Day 1976; Okon et al., 1976) Dobereiner and Day (1976) showed that A.lipoferum can utilize glucose and sucrose as sole carbon sources. But glucose and sucrose are poor carbon sources for A.brasilense (Tarrand et al., 1978). Azospirillum
does not use sucrose directly but host plant roots transform sucrose and other plant products into subtrates suitable for

The typical nitrogen fixing strains of S.lipoferum are 'V' shaped (vibroid) or helical or spiral and possess PBH granules. (Tarrand et al., 1978). The spherical 'C' forms of bacteria occur when they grow on plates under air. The 'C' forms show the presence of capsule external to the lipopolysaccharide layer and this distinguishes it from 'V' forms. The 'C' forms show reduced motility, formation of encapsulated clusters and contain more PHB than in the usual 'V' form (Berg et al., 1979; Berg et al., 1980).

Neyra et al, (1977) stated that Azospirillum becomes the first diazotroph reported to exhibit dissimilatory reduction of nitrate. All the A. lipoferum and half of A. brasilense strains studied reduced nitrate to nitrogen. But the nitrate negative (nir-) strains produce nitrite from nitrate which accumulates, instead of producing nitrogen gas.

Taxonomy

The organism was first described by Beijerinck in 1923 and later (1925) he renamed it as Spirillum lipoferum. In Bergey's manual 1957 it is included in Spirillaceae and is described as that "fixes atmospheric nitrogen in partially pure cultures, ie., free from Azotobacter and Clostridium".

Among the many strains of the bacteria known in the earlier periods three groups were established (Okon et al.,1976. a; Sampoiu et al., 1978. Here the main differences between Group I and Group II are in the utilization of glucose as carbon source, requirement for vitamins and weak or no catalase activity. Group II shows more number of polymorph cells. Group III is distinguished from Group I by the inability to reduce NO_2 to gaseous nitrogen.

Later S. lipoferum has been reclassified based on DNA homology studies. A new genus with two species is now recognised and described. The earlier mentioned Group I and Group III strains of S. lipoferum which are nitrate reductase (denitrification) positive and negative are both included in Azospirillum brasilense. They are designated as A.brasilense nir^+ and A.brasilense nir^- respectively. Eventhough these two subgroups could not be identified by DNA homology, they represented distinct immuno flourescent reactions (De polli et al., 1979).

A.lipoferum can utilise glucose and sucrose as sole carbon source. But these are poor carbon source for A.brasilense (Dobereiner and Day, 1976). Tarrand et al (1978) observed that

A.lipoferum causes acidification of glucose based peptone medium under aerobic conditions, may acidify glucose, fructose, ribose or mannitol under anaerobic conditions and requires biotin for growth. A. brasilense is incapable of utilizing glucose, does not acidify glucose, ribose or mannitol and need no vitamins in semisolid NFb with 0.005% yeast extract. Both species grow best on organic acids and can utilize fructose. Tarrad et al., (1987) found that there were differences in cell forms in older cultures among the two species. Later Megalhaes et al., (1987) proposed a third species A. amazonense. It is more acid tolerant and found in the palm tree roots in the Amazon region.

Reinhold et al., (1987) reported a "group of bacteria phenotypically and electrophoretically homogeneous and clearly distinct from A. amazonense, A. lipoferum and A. brasilense. There was no DNA-DNA binding with any of the three species above". They proposed a fourth Azospirillum species for this group of isolates. These isolates showed better growth at increased NaCl concentrations and named it as A. halopraeferens.

Khammas and a Kaiser (1991) reported a fifth and new Azospirillum sp, A. irakense, nitrogen fixing and pectinolytic bacterium found associated with roots and rhizosphere of rice in Diwaniya, Iraq. This species produces pectatelyase and pectin methyl esterase and can fix nitrogen when pectin is the sole carbon source. The four other species mentioned earlier failed to show pectinolytic activities.

Nitrogen fixation

The ability of Azospirillum to fix nitrogen has been confirmed by many workers using microkjeldhal assay, acetylene reduction assay (ARA) and ^{15}N isotope studies (Kumari et al., 1976; Okon et al., 1976.b; Depolli et al., 1979). Azospirillum grows aerobically in the presence of combined source of nitrogen without any fixation and oxygen concentration is one of the key aspects of nitrogen fixation by the bacteria.

Azospirillum fixes nitrogen only under microaerophilic conditions.Dobereiner and Day (1976) reported nitrogen fixation values as high as 115mg N/g of lactate as carbon source. Nitrogen fixation of the bacteria was found to be in the range of 20 and 24 mg N/g of carbon source (Okon et al., 1976). Nelson and Knowles (1978) observed that nitrogen fixation was between 4.7 and 28 mg N/g of carbon source. Dobereiner (1983) noticed that the efficiency of nitrogen fixation increases with increasing age of the culture. The nitrogen fixation efficiency was 92 mg N/g of 8lactate and 49 mg N/g of glucose for A.brasilense and A.lipoferum respectively in an early stationary phase. Nitrogen fixation by Azospirillum is limited to microaerophilic conditions. S.lipoferum has very poor oxygen protection mechanism and hence cannot grow in air using N_2 as its nitrogen

source. A partial pressure of oxygen above 0.005 atm inhibited nitrogenase activity. Azosp irillum is aerotactically attracted to microaerophilic conditions even when they are not fixing nitrogen. Perhaps this may be the reason why microaerophilic habitat provided by plant roots attracts Azospirillum. In laboatory culture of Azospirillum the simplest and best method of providing a proper oxygen concentration for optimum nitrogen fixation is to provide a semi solid medium for growth (Okon et al., 1976).

The nitrogenase activity of the organism is dependent on many factors such as soil pH, temperature, oxygen concentration etc. (Watanabe and Kuk Ki Lee, 1977). Studies on Nitrogen fixation in different crop plants showed that some quantity of nitrogen could be saved on inoculation with Azospirillum. Smith et al. (1976) recorded a saving of 32kg N/ha in pearl millet. Ruschel and vose (1977) found that about 17% of plant nitrogen in sugarcane was derived from the nitrogen fixation. In Bermuda grass (Cynadon dactylon) total nitrogen content increased by 13% and nitrogen content of top growth increased by 21% over control. (Baltensperger et al., 1978). Bouten and Zuberer (1979) found that Brachiaria, Paspalum, Cynadon and Adropogan species showed an increase upto 33kg N/ha/year by ARA. Field trials by Pal and Malik (1981) showed that in grain sorghum inoculated with

A.brasilense the contribution of nitrogen was between 5.8 and 19.6kg/ha. In uninoculated maize Von Bulow and Dobereiner (1975) observed a potential nitrogen fixation of 2kg N/ha. Lower nitrogen fixation rates were noted in rice by Nayak et al. (1986) and Rao and Rao (1983).It was suggested by Van Berkum and Sloger (1983) that in wild rice a declining carbon reserves and increasing levels of ammonium would certainly restrict nitrogenase activity. The inoculation wth Azospirillum increased the nitrogenase activity temporarily but the increase became insignficant as the rice plants grew older (Rao and Rao, 1983; Watanabe and Lin, 1984).

p^H requirement of Azospirillum

The occurrence of S.lipoferum in soils is highly pH dependent. Eventhough Azospirillum is sensitive to low pH, its occurrence is found associated with plants in soils where the pH (<5.5) is not favourable for its growth. The pH around 7.0 was optimum and its sporadic occurrence in soil with pH 4.8 had been observed (Dobereiner and Day, 1976). The presence of S.lipoferum in different rice soil types like alluvial, laterite and pokkali and kari soils had been reported (Rao et al., 1978). Azospirillum occurs in the acid soils with pH as low as 3.2

(Charyulu and Rao, 1980). Alkaline and saline soils with pH 8.0 to 8.8 also showed presence of the bacteria (Purushothaman and Oblisami, 1985).

As in the case of any enzyme the requirement of pH for nitrogenase activity of Azospirillum also is very narrow. The maximum activity occurs between a pH of 6.8 and 7.8. The nitrogen fixation was reduced considerably at pH of 5.5 and above 8.0 (Dobereiner and Day, 1976). Optimum growth and acetylene reduction by the bacteria occurred between a pH range of 7.0 and 7.5 (Day and Mishra 1983).

Phytohormone production

Research reports confirm that many Azospirillum strains produce plant hormones in broth culture. A number of growth substances are produced by the bacteria. These include IAA, gibberillins and cytokinin like substances (Tien et al.,1979). The phytohormones produced cause root hair multiplication and shortening and thickening of roots in monoxenic cultures (Umali and Garcia, 1979). The production of Indole acetic acid and tryptophan by A.brasilense was reported by Reynders and Vlassak (1979). According to Tien et al (1979) A.brasilense produced IAA and Indole lactic acid from tryptophan. They noticed that IAA

production increased from 1-100 μ g ml^{-1} when tryptophan concentration was increased and the phytohormone production was maximum at the stationary growth phase of bacteria. Hartmann et al. (1983) reported IAA production of 16 μ g ml^{-1} in broth culture. A.brasilense was reported to produce IAA to the extent of 26.1 μ g ml^{-1} in broth culture (Crozier et al., 1988).

Fallik et al. (1989) observed the production of IAA and IBA by A.brasilense in maize. Many other scientists also reported production of IAA by Azospirllum (Jain and Patriquin, 1985; Barbieri et al., 1986; Ruckdaschel et al., 1988). Horemans et al (1986) found that no Gibberillins was produced by Azospirillum. Bottini et al (1989) reported the production of Gibberillins by A.lipoferum. They found that A.lipoferum cultures produced GA3 equivalent to 20-40 pg ml^{-1} of broth. The production of many other hormones in a comparatively lower levels were also observed. These include Indole-3-ethanol, Indole-3-methanol(Crozier et al., 1988), unidentifiable indole derivatives (Baschan and Levamony, 1990), several Gibberillins (Bottini et al., 1989; Tien et al., 1979), Abscisic acid (Kolb and Martin, 1985) and Cytokinins (Horeman et al., 1986; Tien et al., 1979).

Inoculation effects

The inoculation with Azospirillum influences root parameters positively. This include the increase in root

length .p158particularly in the root elongation zone. Increased cell division in the merismatic region of inoculated plants resulted in better root elongation. Moreover the inoculated plants showed increase in number of lateral roots with dense root hairs, root volume and root dry weight. (Dewan and Subba Rao, 1979).

The plant responses as a result of inoculation with Azospirillum were attributed primarily to the production of plant growth promoting substances. (Gaskins and Hubbell 1979). The root exudates contain tryptophan which acted as precursor for IAA synthesis by Azospirillum. The root hairs and lateral root proliferation were increased considerably on inoculation. The excretion of IAA, gibberillins and cyokinins by A.brasilense was also reported. (Kapulnik et al., 1985 a,b; Kolb and Martin, 1985; Barbieri et al., 1986,1988; Morgenstern and Okon, 1987). A stimulation in the root exudation was noticed by Heulin et al., 1987; Lee and Gaskins, 1982). Christiansen and Weniger (1988) found that plant hormones produced on inoculation influence the nitrogen fixing capacity of Azospirillum positively.

The Azospirillum inoculation had significant influence on many foliage parameters and growth characteristics. The inoculation help plants in better mineral uptake especially No3-, NH_4^+, PO_2^{++}, K^+, Rb^+ and Fe^{2+}. This resulted in increased

accumulation of these minerals in stems and leaves and thus an increase in total dry matter. During the reproductive phase this serve as additional source of nutrients and fnally result in higher yield. (Barton et al., 1986; Jain and Patriquin, 1985; Kapulnik et al., 1985 b; Morgenstern and Okon, 1987). It was suggested by Morgenstern and Okon, 1987 that the increased mineral uptake by plants may be due to the positive effects of inoculation on the root parameters.

The positive effects of Azospirillum inoculation on the growth and yeild of rice had been proved by many research workers. Dewan and Subba Rao (1979) reported an increase in the seedling root biomass of rice. Karthikeyan (1981) found that inoculation increased the plant height and dry matter accumulation in rce. Plant bacterisation by root dipping and soil application of Azospirillum caused early tillering and reproductive growth on wet land rice. But there was no increase in total dry weight. (Watanabe and Lin, 1984). Gopalaswamy and Vidyasekaran (1987) confirmed after a field experiment that split application of Azospirillum through seed, seedling and soil increased the plant height. Seed and soil inoculated rice showed increased tillering (Murali and Purushothaman, 1987).

Azospirillum inoculation through seed and soil in the nursery significantly increased the seedling height, number of leaves, dry weight of shoot and root and root length (Subramonian,1987). Gopalaswamy et al. (1989, a) revealed that Azospirillum application didnot increase the plant height significantly when inoculated through seed and seedling root dip. The effect of inoculation on total yield increase in field usually ranged from 10 to 30% (Kapulnik et al., 1981 c, 1987; Rao et al., 1983; Watanabe and Lin, 1984). Grain yield of rice was increased significantly at O, 40 and 60 kg N/ha on inoculation (Subba Rao et al., 1979). The root inoculation of seedlings with Azospirillum resulted in increased grain and straw yields especially at low levels of nitrogen (30 and 45 kg/ha) rather than at 60 kg/ha (Rao et al., 1983). According to Prasad and Singh (1984) bacterisation of seedling with Azospirillum and application of nitrogen increased panicle weight, grains per panicle and protein content.

Inoculation with the bacteria increased the percentage of filled grains and total grain yield per plant. (Watanabe and Lin, 1984). The application of Azospirillum had a significant effect on grain and straw yields at all nitrogen levels. But 50% nitrogen level gave maximum yield on inoculation (Balasubramonian and Kumar (1987). Seedling root dip inoculation resulted in increased grain yield and nitrogen uptake at all levels of

nitrogen (Prasad and Singh, 1984). According to Balasubramonian (1987) the grain yield obtained with 50% nitrogen and Azospirillum inoulation was on par with 100% nitrogen alone. Govindaswamy et al. (1992) reported an increase in total grain and straw yield of rice on Azospirillum inoculation.

CHAPTER III

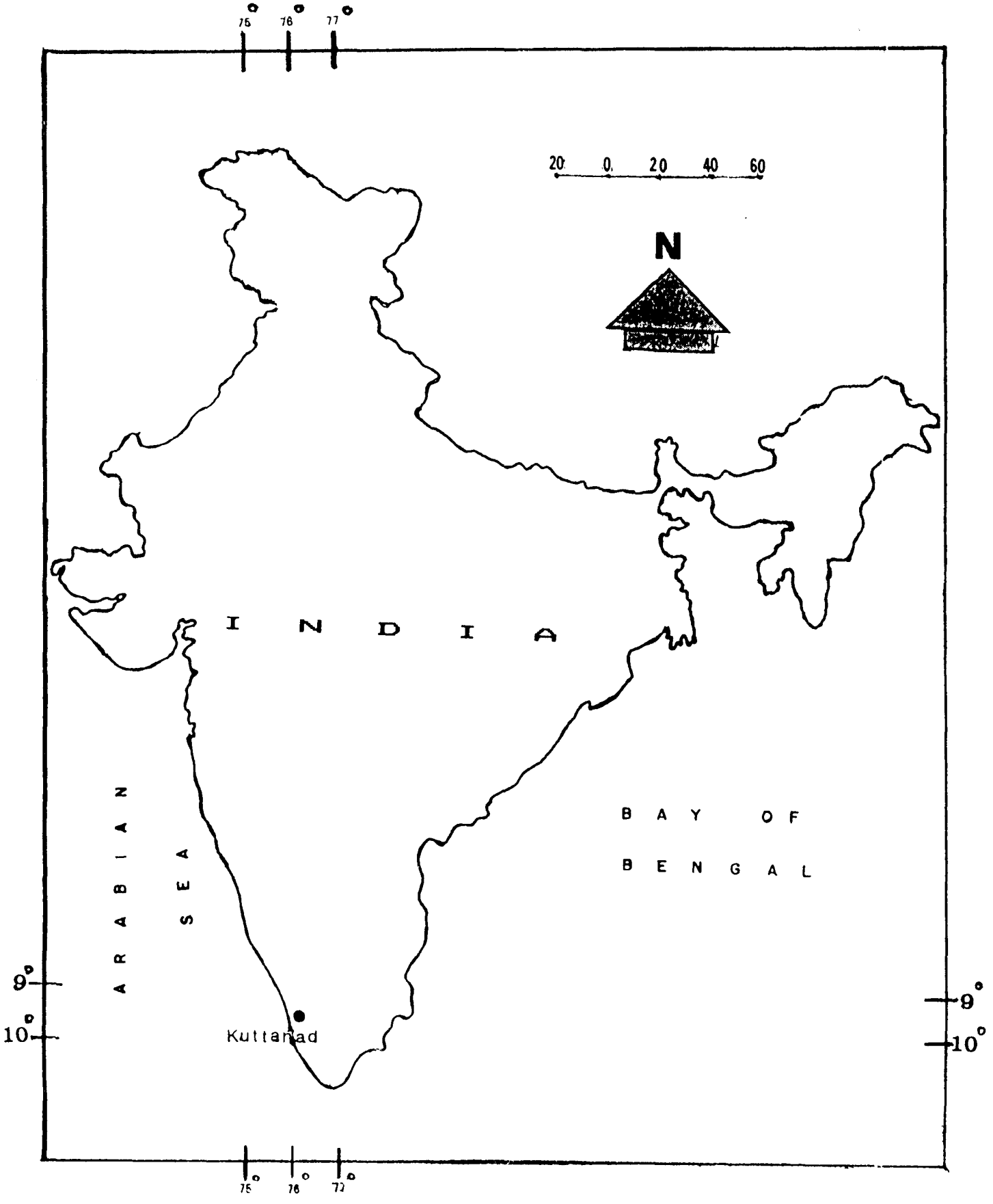
MATERIALS AND METHODS

The present investigation was intended to isolate acid and salt tolerant strains of Azospirillum and to develop inoculant cultures suited for rice in the acid sulphate soils of Kuttanad, Kerala. Fifty root samples of rice seedlings (20-25 DAT) were collected from twentyfive different locations, with different soil pH, in Kuttanad. The root samples were washed thoroughly and preserved in polythene bags for the isolation of Azospirillum. On each day about five samples were collected from the field and they were isolated on the following day. The location of collection of samples are given in the table 1.

3.1 Isolation and purification of Azospirillum from roots of rice

The isolation of Azospirillum was done following the method of Bulow and Day (1975). The roots were cleaned of all adhering soil and other debris in clean tap water and later washed thoroughly with sterile water. The sample was then cut into pieces of 0.5cm length, surface sterilised with 1% choloromine-T solution for 15 minutes followed by serial washing in sterile water 4 or 5 times and finally in the phosphate buffer (PH 7.0).

Fig.1. Location map of the area of study.



The surface sterilised bits were aseptically transferred to test tubes containing Semi solid nitrogen free Bromothymol blue medium (Appendix 1). After 48-72 h of incubation at 37°C, appearance of characteristic undulating white pellicle just below the surface of medium indicated the presence of Azospirillum. The colour of the medium changed from yellowish green to blue. From the positive tubes a loop full of the culture was transferred to fresh semisolid malate medium and purified by three serial transfers. Final purification was done by streaking diluted cultures in potato infusion agar (Appendix 1). Single dry wrinkled colonies that appeared on the plates were transferred to semi solid NFb after 3 days incubation. The formation of white subsurface undulating pellicle confirmed successful isolation of Azospirillum. The pure cultures were streaked finally on potato sucrose agar slants and stored at 4°C for further use.

3.2 Characterisation of the isolates

3.2.1 Morphology

The isolates of Azospirillum were grown in semi solid malate medium in screw cap bottles. Gram's staining was carried out according to Hucker's modification.(Frobischer,1964). The shape and size of bacteria were studied in 72 hours old culture.

3.2.2 Physiological test.

3.2.2.1 Utilization of different carbon sources

The purified isolates of Azospirillum were tested for utilization of three different carbon sources namely glucose, fructose and sucrose. Semisolid Nfb was prepared by replacing malic acid with 0.5% filter sterilized carbon source under study. The inoculation was done with 48 h old culture and tubes were incubated at 37^{o} C for 72 h and formation of undulating subsurface pellicle indicated the utilization of the carbon source added in the medium.

3.2.2.2 Biotin requirement:

The glasswares were cleaned thoroughly with distilled water and baked in an oven to destroy even the traces of biotin present. Nitrogen free semi solid malate medium was prepared with and without biotin (0.0001g l^{-1}). The medium was inoculated with 48 h old Azospirllum culture and incubated for 48 hours at 37^{o} C. In cases where growth occurred in the medium without biotin a second transfer was made to fresh medium with and without biotin to confirm its biotin requirement.

3.2.2.3 **Acidification of peptone based glucose medium**

The medium with following composition described by Kreig and Dobereiner (1984) was used to detect the production of acid by isolates:

Peptone	-	2g
MgSo4 $7H_2O$	-	1g
$(NH4)_2SO_4$	-	1g
$FeCl_3$ $6H_2O$	-	0.002g
$MnSO_4$ H_2O	-	0.002g
Bromothymol blue in dilute KOH	-	0.025g

This was prepared in 950ml distilled water and the pH was adjusted to 7.0 and sterilized by autoclaving. After cooling 50ml of 20% W/V (weight/volume) of filter sterilized glucose was added aseptically. The tubes were inoculated with fresh cultures of Azospirillum. The development of yellow colour after incubation for 48-96 hours indicated acidification.

3.2.2.4 **Catalase activity**

A loopful of fresh Azospirillum culture from semi solid malate medium was taken on a clean glass slide. One ml of

hydrogen peroxide was poured over it. A brisk effervescence indicated the presence of catalase activity in the culture.

3.2.2.5 **Salt dependence**

Semi solid Nfb was prepared with double glass distilled water with and without 0.25% Nacl. Fresh culture of Azospirillum was used to inoculate 5 ml of the medium and observed for growth after 48 hours of incubation to verify whether the isolates were salt dependent. The isolates which failed to grow in salt deficient medium but grew in NaCl supplemented medium were considered as salt dependent.

3.2.2.6 **Salinity tolerance**

Semi sold Nfb was prepared and sodium chloride (Nacl) was added so as to get final concentrations of 0.5.1 ,2,3, and 4%. The medium was sterilized by autoclaving and fresh culture of Azospirillium was used to inoculate 5 ml of the medium. The maximum level of tolerance of Azospirillum to NaCl concentration was recorded.

3.2.2.7 **Acid tolerance**

Semi solid Nfb was prepared and the pH was adjusted to different values (3.5, 4.3, 4.2, 4.1, 4.5, 5.0, 5.0 and 6.0). The exact pH after autoclaving was noted by checking the parallel

set of tubes maintained. Fresh culture of Azospirillum was used to inoculate 5 ml of the medium. The tubes were incubated at 30° C. Observations were made for the appearance of white pellicle just below the surface which indicated the presence of growth at that particular pH.

3.3. Production of Indole Acetic Acid (IAA) in vitro

Nitrogen free malate broth was prepared without addition of bromothymol blue indicator and 100ml of the broth was taken in 250ml flasks. After sterilization, freshly prepared filter sterilized L-tryptophan solution was added so as to get a final concentration of 100 Ug ml^{-1}. Fresh Azospirillum culture was used to inoculate the flask and incubated for 7 days in the dark. The extraction and estimation of IAA from culture filtrate were done following the method of Chandramohan and Mahadevan (1968). The cultures, after 7 days incubation, were centrifuged to remove the bacterial cells and the supernatant was acidified to pH 3 with 1N HCl. IAA was extracted with equal volumes of ehtyl ether in a separating funnel at 4° C. The extraction was repeated thrice using 100ml ehtyl ether for each extraction. The ether extracts were pooled and ether was flash evaporated at 35-40° C and the residue was dissolved in 2 ml methanol. IAA in the methanol fraction was quantitatively measured using Salper reagent (Salper reagent is prepared as 1ml

0.5 M ferric chloride in 50ml 30% perchloric acid). To 1.5 ml distilled water in a test tube 0.5ml methanol residue was mixed and 4ml of Salper reagent was rapidly added. The samples were incubated in dark for 1 hour for maximum colour development. The colour intensity was read at 535nm in spectronic 20 spectrophotometer. The quantity of IAA in the culture filtrate was calculated from the standard curve.

3.4 Estimation of *in vitro* Nitrogen fixation

Semisolid Nfb supplemented with 50 mg yeast extract was taken in 100 ml quantities and 1 ml of inoculum was added. After 7 days of incubation, the medium was homogenised in a waring blender and 5 ml aliquot was withdrawn and digested with 5 ml concentrated H_2SO_4. At the end of digestion, 1 ml $HClC_4$ was added and digestion continued until the contents were clear. After cooling, the aliquot was transferred to a microkjeldhal flask and the total nitrogen was estimated (Humphries, 1956). The result was expressed as mg of nitrogen fixed per g of malate.

3.5 Root elongation studies in rice seedlings

The study was conducted in germination paper rolls, (Anonymous, 1967), Glass tubes containing standard seed germination paper with a fold at top were covered with aluminum

foil, wrapped in paper covers and sterilized by autoclaving The seeds were surface sterilized with 0.1% mercuric chloride for 45 S and there after washed thoroughly in three to four changes of sterile water. They were soaked in the Azospirillum cultures for one hour and the inoculated seeds were germinated on soft water agar (1% weight/volume) in a sterile petriplate for two days. Three uniformly germinated seeds were aseptically placed on the folds of the germination paper so that the radicle faced downwards in the perforation made by a sterile forceps and 30 ml of nitrogen free nutrient solution was poured along the side of the tubes with least disturbance to the seeds. The tubes were protected from other disturbances in a screen house. Half strength nitrogen free nutrient solution was given to keep germination paper always moist. After 15 days the paper rolls were removed, unfolded and the root lengths measured.

The most efficient cultures were selected based on nitrogen fixation, IAA production, root elongation, and acid and salt tolerance.

3.6 Screening of the selected Azospirillum isolates for biomass production in rice

The experiment was conducted under green house conditions. Medium sized and uniform earthen pots were filled with 3 kg Kari soil from Karumadi, Kuttanad.The treatments included combinations

of four isolates of Azospirillum (AZR 15,22,37 and 43) with graded levels of nitrogen(N1-No nitrogen, N2 -50%, N3-75% and N4-100% of recommended dose. Other fertilizers were given as per the package of practices recommendations. There were 3 replications in the experiment conducted in CRD. Surface sterilized and sprouted seeds of rice variety "Jyothy" were soaked for one hour in cell suspension of Azospirillum isolate. Organic manure at the rate of 7.5 g per pot was incorporated into the soil. The inoculated rice seeds were sown in the pots separately and labelled properly. The nursery pots were irrigated as and when necessary. After 20 days of growth the seedlings were carefully uprooted and adhering soil washed off. The root system was dipped in the respective Azospirillum culture suspension for 12 h and transplanted into the pots. Full amount of phosphorous and 2/3 nitrogen and 1/2 potassium of package of practices recommendations were applied basally,. The remaining parts of the fertilizers were applied at the panicle initiation stage. The water level was maintained at 3cm. The pots were kept weed free throughout crop growth period by hand weeding. BHC 50% WP was applied at 0.2% to control the incidence of rice leaf folder. Minor incidence of brown spot disease was noticed which was not significant.

The following biometric observations were made. Plant height in individual pots was recorded at 25 50, 75, 100 days after transplanting (DAT) and at harvest. The total number of tillers in each hill was recorded at 25, 50, 75 DAT and productive tillers at 85 DAT. AT harvest the weight of sun dried straw and length of roots were noted. Total grain yield and 1000 grain weight were determined from sun dried samples. The root weight was determined after oven drying of washed roots at 85° C for 24h to constant weight and dry weight expressed as mg plant^{-1}

3.7 Chemical analysis of plant and soil and computation of nitrogen balance.

Plant samples taken at harvest were dried in oven at 70°C, ground and passed through 0.5 mm mesh. Total nitrogen content of plants was estimated by modified microKjeldhal's method. (Jackson, 1973). The protein content of grains was obtained by multiplying the percentage nitrogen content in grain by the factor 6.25 (Simpson et al, 1965). Soil samples were taken from each pot, after harvest, dried in shade, powdered and seived through 2 mm sieve and the nitrogen content was estimated by microKjeldhal method (Jackson, 1973).

3.8 Nitrogen balance studies

The total nitrogen content of soil before and after the crop and that removed by the crop have been estimated from the chemical analysis data. This information was made use of to compute the nitrogen balance and the quantity of nitrogen fixed biologically.

3.9 Analysis of the genetic basis of salt tolerance.

In order to study whether the salt tolerance is plasmid borne, the plasmid curing studies were conducted. The isolates 8 and 29 which showed salt tolerance upto 4% sodium chloride were selected for the genetic analysis.

3.9.1 Acridine orange curing

The plasmid curing of the isolates was done using acridine orange and sodium dodecyl sulphate. The sterile broth was inoculated with actively growing cells of Azospirillum and incubated for 8-12 hours at 30° C. The filter sterilized acridine orange was added in the medium to get a final concentration of 0, 5, 10 and 20 ppm. The culture was again incubated for another 12h at 30° C. The serially diluted cultures were plated in malate medium without NaCl so as to get

100-150 colonies per plate. The colonies were then replica plated in malate medium with 4% Nacl to study the salt tolerance. The number of cured colonies, as indicated by failure of growth, counted and expressed as per cent curing. (Mahadeven, 1976).

3.9.2 Sodium dodecyl sulphate curing

The SDS curing of plasmids of isolates was done following the method of Salsbury et al. (1972). To 100 ml of the broth 1g SDS was added and the solution was autoclaved. The ^{pH} of solution was adjusted to 7.6 after autoclaving and was steamed for 1 h. One ml of the stationary culture of Azospirillum was added to the broth and incubated for 24 hrs. Then the culture was plated on malate medium without Nacl prepared using double glass distilled water. The colonies were replica plated for salt tolerance in 4% NaCl containing malate medium and the number of cured colonies expressed in percentage.

CHAPTER IV

RESULTS

The results of the various studies conducted under the investigation are given here.

4.1 Isolation of Azospirillum

The isolation of Azospirillum was made from 25 different locations following the procedure described in the meterials and methods. Fifty isolates representing the 25 locations are given in the table 1

4.2 Characterisation of the isolates .

4.2.1 Morphological characters.

The morphological characters of the isolates like size, shape, motility and presence of poly beta hydroxy granules were observed in 72 h old cultures grown in semi solid malate medium. The isolates were small to medium sized rods and showed slow motility. Even though polymorphism was noted, majority of isolates were curved rods. The poly beta hydroxy granules were present in all the isolates. All the isolates retained counterstain safranin indicating that they were Gram negative.

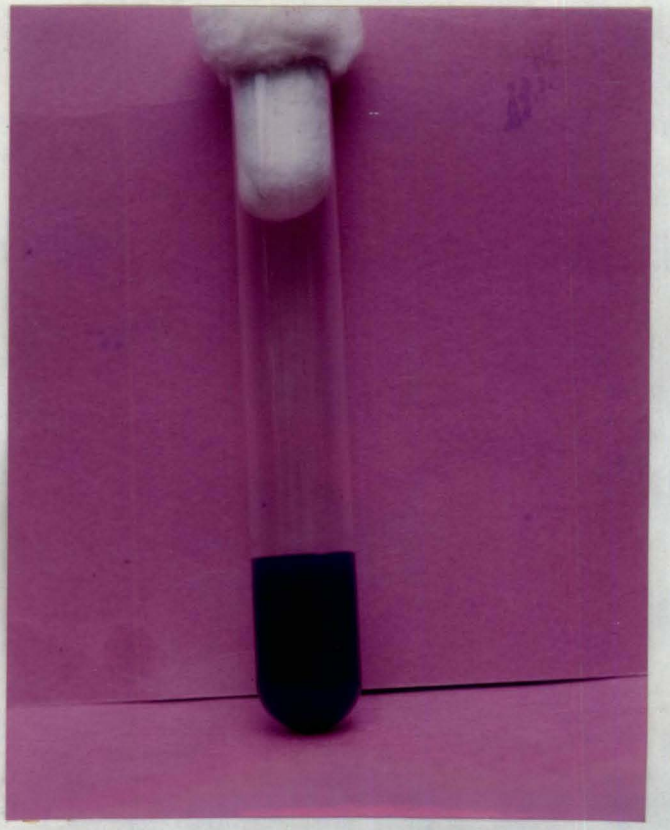


Plate.1 A test tube containing semi solid NFb medium showing white pellicle of *Azospirillum* below the meniscus.

Plate.2 A close up view of the Azospirillum Pellicle formation.

Table:1 Locations of Collection of rice root samples for isolation of *Azospirillum*.

Location	Isolate
Tharayakkari North	AZR 1, AZR 2
Tharayakkari South	AZR 3, AZR 4
Karuvatta	AZR 5, AZR 6
Kochuputhenkari	AZR 7, AZR 8
Kavilthekkepuram	AZR 9, AZR 10
Cheriyathuruthu	AZR 11,AZR 12
Varikattu Kari North	AZR 13,AZR 14
Varikattu Kari South	AZR 15,AZR 16
Kallepadom	AZR 17,AZR 18
Ezhavan Kari	AZR 19,AZR 20
Monkambu	AZR 21,AZR 22
Veliyanad	AZR 23,AZR 24
Ramankari	AZR 25,AZR 26
Niranam	AZR 27,AZR 28
Edathua	AZR 29,AZR 30
Kavalam	AZR 31,AZR 32
Kidangara	AZR 33,AZR 34
Chambakulam	AZR 35,AZR 36
Thakazhi	AZR 37,AZR 38

(Table 1 contd.)

Location	Isolate
Cheruthoni	AZR 39,AZR 40
Kollanad	AZR 41,AZR 42
Punnakunnathusery	AZR 43,AZR 44
Maniyen Karipadam	AZR 45,AZR 46
Muttar	AZR 47,AZR 48
Kalathil Melpathu	AZR 49,AZR 50

4.2.2 Physiological Tests

4.2.2.1 Utilization of Different Cabron sources

The isolated Azospirillum cultures were tested for their ability to utilize three different carbon sources and the data is presented in the table 2.Out of the 50 isolates 20 showed good growth in glucose where as rest failed to grow .All the isolates grew well on fructose .The utilisation of sucrose as the sole carbon source was noticed in some isolates.

4.2.2.2 Biotin requirement.

The requirement of biotin for growth of the Azospirillum isolates was tested in medium with and without biotin (table 3). All isolates showed good growth in biotin containing medium. Thirty isolates showed growth in the absence of biotin.

4.2.2.3 Acidification of glucose medium.

Among the 50 isolates tested 19 developed yellow colour on inoculation indicating good acidification of glucose medium(Table 5)

Table 2 Utilisation of carbon sources by the Azospirillum isolates

Isolate	Carbon sources		
	Glucose	Fructose	Sucrose
AZR 1	-	+	-
AZR 2	+	+	-
AZR 3	+	+	-
AZR 4	-	+	-
AZR 5	-	+	-
AZR 6	-	+	+
AZR 7	-	+	-
AZR 8	-	+	-
AZR 9	+	+	+
AZR 10	+	+	-
AZR 11	-	+	-
AZR 12	+	+	-
AZR 13	-	+	-
AZR 14	-	+	-
AZR 15	-	+	-
AZR 16	+	+	-
AZR 17	-	+	-
AZR 18	+	+	-
AZR 19	+	+	+
AZR 20	-	+	-
AZR 21	+	+	+
AZR 22	+	+	-
AZR 23	-	+	-
AZR 24	+	+	-
AZR 25	-	+	+
AZR 26	+	+	-
AZR 27	-	+	-
AZR 28	+	+	+
AZR 29	-	+	-
AZR 30	-	+	-
AZR 31	-	+	-
AZR 32	+	+	+
AZR 33	-	+	-
AZR 34	-	+	-
AZR 35	-	+	-

Table 2 (contd.....)

Isolate	Carbon sources		
	Glucose	Fructose	Sucrose
AZR 36	+	+	-
AZR 37	-	+	-
AZR 38	+	+	-
AZR 39	-	+	-
AZR 40	+	+	-
AZR 41	-	+	-
AZR 42	+	+	-
AZR 43	-	+	-
AZR 44	-	+	-
AZR 45	-	+	-
AZR 46	-	+	-
AZR 47	+	+	-
AZR 48	-	+	-
AZR 49	-	+	-
AZR 50	+	+	-

+ Pesence of growth
- Absence of growth

Table 3 Requirement of biotin for growth of *Azospirillum* isolates

Isolate	medium with biotin	without biotin
AZR 1	+	+
AZR 2	+	+
AZR 3	+	+
AZR 4	+	+
AZR 5	+	+
AZR 6	+	+
AZR 7	+	-
AZR 8	+	-
AZR 9	+	-
AZR 10	+	+
AZR 11	+	+
AZR 12	+	-
AZR 13	+	+
AZR 14	+	+
AZR 15	+	-
AZR 16	+	-
AZR 17	+	+
AZR 18	+	-
AZR 19	+	+
AZR 20	+	-
AZR 21	+	-
AZR 22	+	-
AZR 23	+	+
AZR 24	+	-
AZR 25	+	+
AZR 26	+	-
AZR 27	+	+
AZR 28	+	-
AZR 29	+	+
AZR 30	+	+
AZR 31	+	+
AZR 32	+	-
AZR 33	+	+
AZR 34	+	-
AZR 35	+	+
AZR 36	+	+
AZR 37	+	+
AZR 38	+	-
AZR 39	+	+

Table 3 (contd....)

Isolate	medium with biotin	without biotin
AZR 40	+	-
AZR 41	+	+
AZR 42	+	-
AZR 43	+	+
AZR 44	+	+
AZR 45	+	-
AZR 46	+	+
AZR 47	+	-
AZR 48	+	+
AZR 49	+	+
AZR 50	+	+

\+ Presence of growth

\- Absence of growth

4.2.2.4 Catalase activity

The catalase activity of the various isolates was studied and the results are presented in table 5. A brisk effervesence on addition of H_2O_2 to culture growth in glass slides was seen in all the isolates. This indicated that all isolates under study were catalase positive.

4.2.2.5.Salt dependence

The salt dependence of the Azospirillum isolates was tested in the absence of sodium chloride (table 4).Of the 50 isolates tested 37 isolates did not show any salt dependence .However 13 of the isolates showed growth only in NaCl added medium and were considered to be salt dependent .

4.2.2.6 Salinity tolerance

The ability of the isolates to tolerate the maximum concentration of sodium chloride was tested and presented in table 6. Most of the isolates showed tolerance up to 2.0% level of sodium chloride concentration. The isolates AZR 2, 6, 8, 13 and 29 tolerated up to 4.0% NaCl. None recorded tolerance beyond 4.0%.

Table 4 Salt dependence of *Azosripillum* isolates

Isolate	Salt dependence
AZR 1	-
AZR 2	-
AZR 3	+
AZE 4	-
AZR 5	-
AZR 6	-
AZR 7	-
AZE 8	+
AZR 9	-
AZR 10	-
AZR 11	-
AZR 12	-
AZR 13	+
AZR 14	-
AZR 15	-
AZR 16	-
AZR 17	+
AZR 18	-
AZR 19	-
AZR 20	+
AZR 21	-
AZR 22	-
AZR 23	+
AZR 24	-
AZR 25	-
AZR 26	-
AZR 27	-
AZR 28	+
AZR 29	-
AZR 30	+
AZR 31	-
AZR 32	-
AZR 33	+
AZR 34	-
AZR 35	-
AZR 36	-
AZR 37	+
AZR 38	-
AZR 39	+
AZR 40	-

Table 4 (contd....)

Isolate	Salt dependence
AZR 41	-
AZR 42	-
AZR 43	-
AZR 44	-
AZR 45	+
AZR 46	-
AZR 47	-
AZR 48	-
AZR 49	+
AZR 50	-

+ Salt dependent
- Salt independent

Table 5 Acidification of glucose medium and catalase activity by *Azospirillum* isolates

Isolate	Acidification of glucose medium	Catalase activity
AZR 1	-	+
AZR 2	+	+
AZR 3	+	+
AZR 4	-	+
AZR 5	-	+
AZR 6	-	+
AZR 7	+	+
AZR 8	-	+
AZR 9	+	+
AZR 10	+	+
AZR 11	-	+
AZR 12	+	+
AZR 13	-	+
AZR 14	-	+
AZR 15	-	+
AZR 16	+	+
AZR 17	-	+
AZR 18	+	+
AZR 19	+	+
AZR 20	-	+
AZR 21	+	+
AZR 22	+	+
AZR 23	-	+
AZR 24	+	+
AZR 25	-	+
AZR 26	+	+
AZR 27	-	+
AZR 28	+	+
AZR 29	-	+
AZR 30	-	+
AZR 31	-	+
AZR 32	-	+
AZR 33	-	+
AZR 34	-	+
AZR 35	+	+
AZR 36	-	+
AZR 37	-	+
AZR 38	-	+

Table 5 (contd...)

Isolate	Acidification of glucose medium	Catalase activity
AZR 39	-	+
AZR 40	+	+
AZR 41	-	+
AZR 42	-	+
AZR 43	-	+
AZR 44	-	+
AZR 45	+	+
AZR 46	-	+
AZR 47	+	+
AZR 48	-	+
AZR 49	+	+
AZR 50	+	+

Table 6 Salinity tolerence by Azospirillum isolates

Isolate	sodium chloride concentration (%)					
	0.5	1.0	2.0	3.0	4.0	4.5
AZR 1	+	+	+	+	-	-
AZR 2	+	+	+	+	+	-
AZR 3	+	+	+	+	-	-
AZR 4	+	+	+	+	-	-
AZR 5	+	+	+	+	-	-
AZR 6	+	+	+	+	+	-
AZR 7	+	+	-	-	-	-
AZR 8	+	+	+	+	+	-
AZR 9	+	+	+	+	-	-
AZR 10	+	+	+	+	-	-
AZR 11	+	+	+	+	-	-
AZR 12	+	+	+	+	-	-
AZR 13	+	+	+	+	+	-
AZR 14	+	+	+	+	-	-
AZR 15	+	+	+	+	-	-
AZR 16	+	+	-	-	-	-
AZR 17	+	+	+	+	-	-
AZR 18	+	+	+	-	-	-
AZR 19	+	+	+	+	-	-
AZR 20	+	+	+	+	-	-
AZR 21	+	+	-	-	-	-
AZR 22	+	+	+	+	-	-
AZR 23	+	+	+	+	-	-
AZR 24	+	+	+	+	-	-
AZR 25	+	+	+	+	-	-
AZR 26	+	+	+	+	-	-
AZR 27	+	+	+	+	-	-
AZR 28	+	+	-	-	-	-
AZR 29	+	+	+	+	+	-
AZR 30	+	+	+	+	-	-
AZR 31	+	+	+	+	-	-
AZR 32	+	+	+	+	-	-
AZR 33	+	+	+	+	-	-
AZR 34	+	+	+	+	-	-
AZR 35	+	+	+	+	-	-
AZR 36	+	+	+	+	-	-
AZR 37	+	+	+	+	-	-
AZR 38	+	+	+	+	-	-

Table 6 (contd....)

Isolate	sodium chloride concentration (%)					
	0.5	1.0	2.0	3.0	4.0	4.5
AZR 39	+	+	+	+	-	-
AZR 40	+	-	-	-	-	-
AZR 41	+	+	+	+	-	-
AZR 42	+	+	+	+	-	-
AZR 43	+	+	+	+	-	-
AZR 44	+	+	+	+	-	-
AZR 45	+	+	+	+	-	-
AZR 46	+	+	+	+	-	-
AZR 47	+	+	-	-	-	-
AZR 48	+	+	+	+	-	-
AZR 49	+	+	+	+	-	-
AZR 50	+	+	+	+	-	-

\+ Presence of growth
\- Absence of growth

4.2.2.7 Acid tolerance

The ability of the isolates to tolerate low pH was tested and the pertaining data is given in table 7. All the isolates showed growth upto pH 5.0. Of the total 50 isolates 19 grew at pH 4.3. But at pH 4.0 only isolates 9,18,21,26 and 35 showed growth and none recorded growth beyond pH 4.0.

4.3 IAA production

The data regarding the production of Indole Acetic Acid by the isolates (Table 8) showed wide variations. Isolate AZR 37 recorded the maximum value of 55 µg l^{-1} whereas isolate 32 gave the minimum IAA production of 24 µg l^{-1}. Most of the isolates showed IAA production around 35 µg l^{-1}.

4.4 Nitrogen fixation

The isolates were tested for their efficiency to fix nitrogen in <u>in vitro</u> (Table 8). The highest fixation was shown by AZR 15 which recorded a value of 21.28mg N/g malate. The isolates AZR 12,22,26,37 and 43 recorded nitrogen fixation of 20.14 mg N/g malate. However the ability to fix nitrogen by the isolates ranged between 11.20 and 21.2 mg N/g malate.

Table 7 Acid Tolerance by *Azospirillum* isolates

Isolates	pH of the medium				
	6.0	5.0	4.5	4.3	4.0
AZR 1	+	+	+	-	-
AZR 2	+	+	+	+	-
AZR 3	+	+	+	-	-
AZR 4	+	+	+	-	-
AZR 5	+	+	+	+	-
AZR 6	+	+	+	-	-
AZR 7	+	+	+	-	-
AZR 8	+	+	+	+	-
AZR 9	+	+	+	+	+
AZR 10	+	+	+	+	-
AZR 11	+	+	+	-	-
AZR 12	+	+	+	+	-
AZR 13	+	+	+	-	-
AZR 14	+	+	+	-	-
AZR 15	+	+	+	+	-
AZR 16	+	+	+	-	-
AZR 17	+	+	+	+	-
AZR 18	+	+	+	+	+
AZR 19	+	+	+	+	-
AZR 20	+	+	+	-	-
AZR 21	+	+	+	+	+
AZR 22	+	+	+	-	-
AZR 23	+	+	+	-	-
AZR 24	+	+	+	-	-
AZR 25	+	+	+	-	-
AZR 26	+	+	+	+	+
AZR 27	+	+	+	-	-
AZR 28	+	+	+	-	-
AZR 29	+	+	+	+	-
AZR 30	+	+	+	+	-
AZR 31	+	+	+	-	-
AZR 32	+	+	+	-	-
AZR 33	+	+	+	-	-
AZR 34	+	+	+	-	-
AZR 35	+	+	+	-	+
AZR 36	+	+	+	-	-
AZR 37	+	+	+	+	-
AZR 38	+	+	+	+	-

Table 7 (contd .....)

Isolates	pH of the medium				
	6.0	5.0	4.5	4.3	4.0
AZR 39	+	+	+	-	-
AZR 40	+	+	+	-	-
AZR 41	+	+	+	-	-
AZR 42	+	+	+	+	-
AZR 43	+	+	+	+	-
AZR 44	+	+	+	-	-
AZR 45	+	+	+	+	-
AZR 46	+	+	+	-	-
AZR 47	+	+	+	-	-
AZR 48	+	+	+	-	-
AZR 49	+	+	+	-	-
AZR 50	+	+	+	-	-

\+ Presense of growth

\- Absence of growth

4.5 Root elongation studies

The influence of Azospirillum inoculation on rice root elongation was studied (Table 8). The inoculated seedlings showed better root length. The isolates which produced better results were AZR 15 (13.8cm), AZR 21 (13.1 cm), AZR 22 (12.7) cm) and AZR 37 (14.2 cm). The control recorded root length of 10.2cm.

4.6 Screening of the selected Azospirillum isolates

The isolates AZR 15,22,37 and 43 selected based on in vitro nitrogen fixing efficiency, IAA production and root elongation (Fig.1,2 and 3) were subjected to pot culture studies. A CRD with 3 replications was laid out to assess the effects of inoculation with Azospirillum isolates and 4 graded levels of fertilizer nitrogen. The data on plant height at 25,50,75,100 DAT and harvest are presented below.

Table 8 Nitrogen fixation, IAA production and root elongation by *Azospirillum* isolates.

Isolate	Nitrogen fixation mg/g malate	IAA production mg/l	Root elongation cm.
AZR 1	11.20	27	9.5
AZR 2	14.00	39	12.3
AZR 3	11.20	46	10.3
AZR 4	15.68	44	9.5
AZR 5	19.04	48	10.2
AZR 6	12.88	38	11.8
AZR 7	11.20	44	10.2
AZR 8	11.20	43	9.8
AZR 9	12.32	48	10.6
AZR 10	11.76	37	8.7
AZR 11	14.00	46	11.1
AZR 12	20.14	34	10.6
AZR 13	15.68	37	12.1
AZR 14	12.32	47	11.9
AZR 15	21.28	50	13.8
AZR 16	12.88	49	12.8
AZR 17	15.68	25	9.7
AZR 18	14.00	30	10.9
AZR 19	11.76	35	11.9
AZR 20	11.76	39	12.3
AZR 21	11.20	48	13.1
AZR 22	20.14	48	11.9
AZR 23	11.20	39	12.7
AZR 24	14.00	40	11.6
AZR 25	15.68	42	12.4
AZR 26	20.14	24	11.4
AZR 27	16.80	32	10.1
AZR 28	19.04	48	9.1
AZR 29	12.32	37	9.8
AZR 30	11.20	35	10.2
AZR 31	11.20	38	8.8
AZR 32	12.32	24	11.2
AZR 33	16.80	32	11.0
AZR 34	11.20	49	12.3
AZR 35	15.68	27	12.1
AZR 36	12.32	39	11.9

Table 8 (contd...)

Isolate	Nitrogen fixation mg/g malate	IAA production mg/l	Root elongation cm.
AZR 37	20.14	55	14.2
AZR 38	16.80	25	10.5
AZR 39	11.76	33	9.9
AZR 40	12.32	37	10.2
AZR 41	11.20	30	12.3
AZR 42	11.76	28	12.0
AZR 43	20.14	49	12.9
AZR 44	11.20	40	9.9
AZR 45	13.34	30	10.6
AZR 46	14.00	32	8.8
AZR 47	12.88	29	11.1
AZR 48	11.20	37	10.2
AZR 49	12.32	40	9.7
AZR 50	11.76	38	9.9

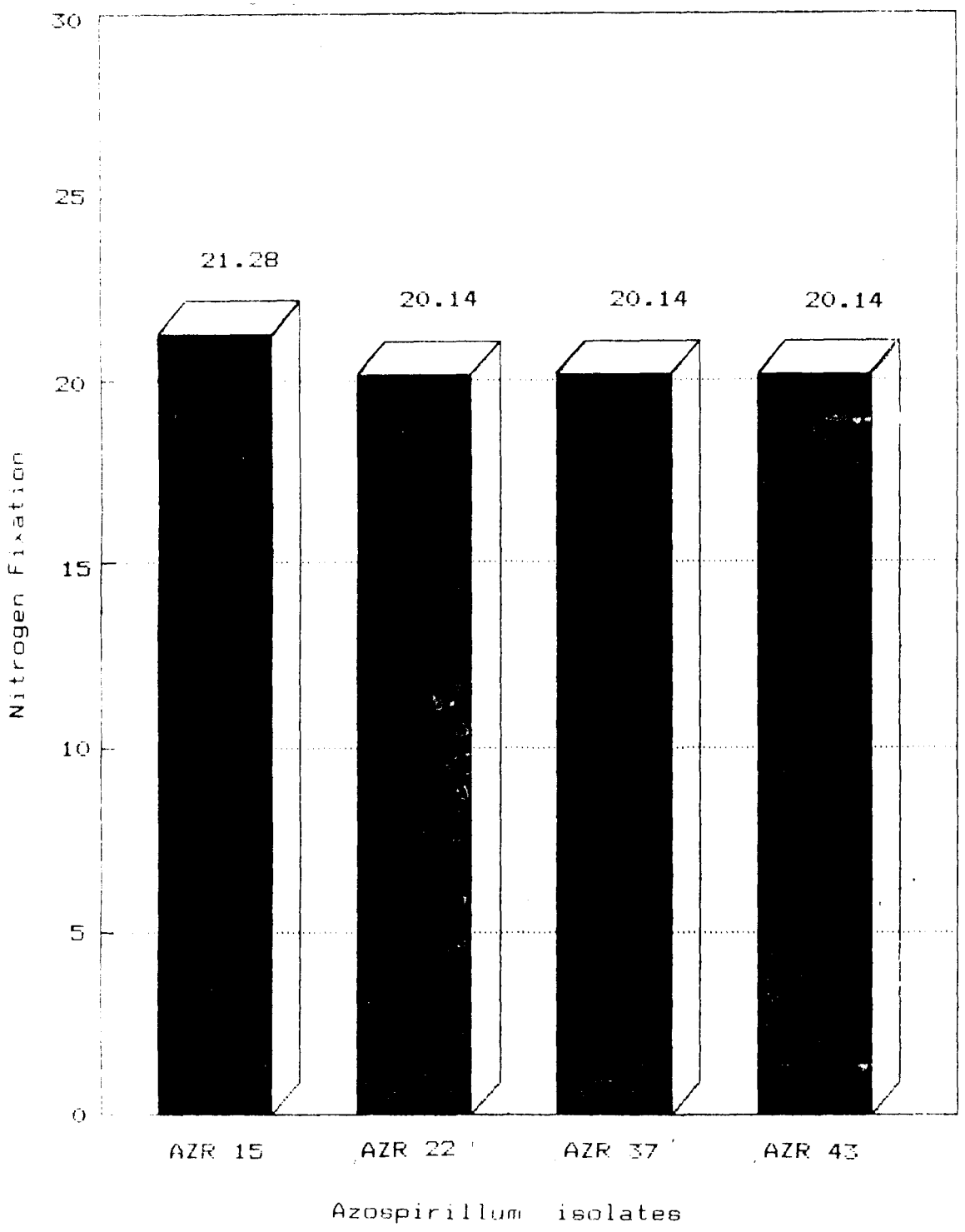


Fig.2. Nitrogen Fixation (mg/g malate) *in vitro* by the selected *Azospirillum* isolates.

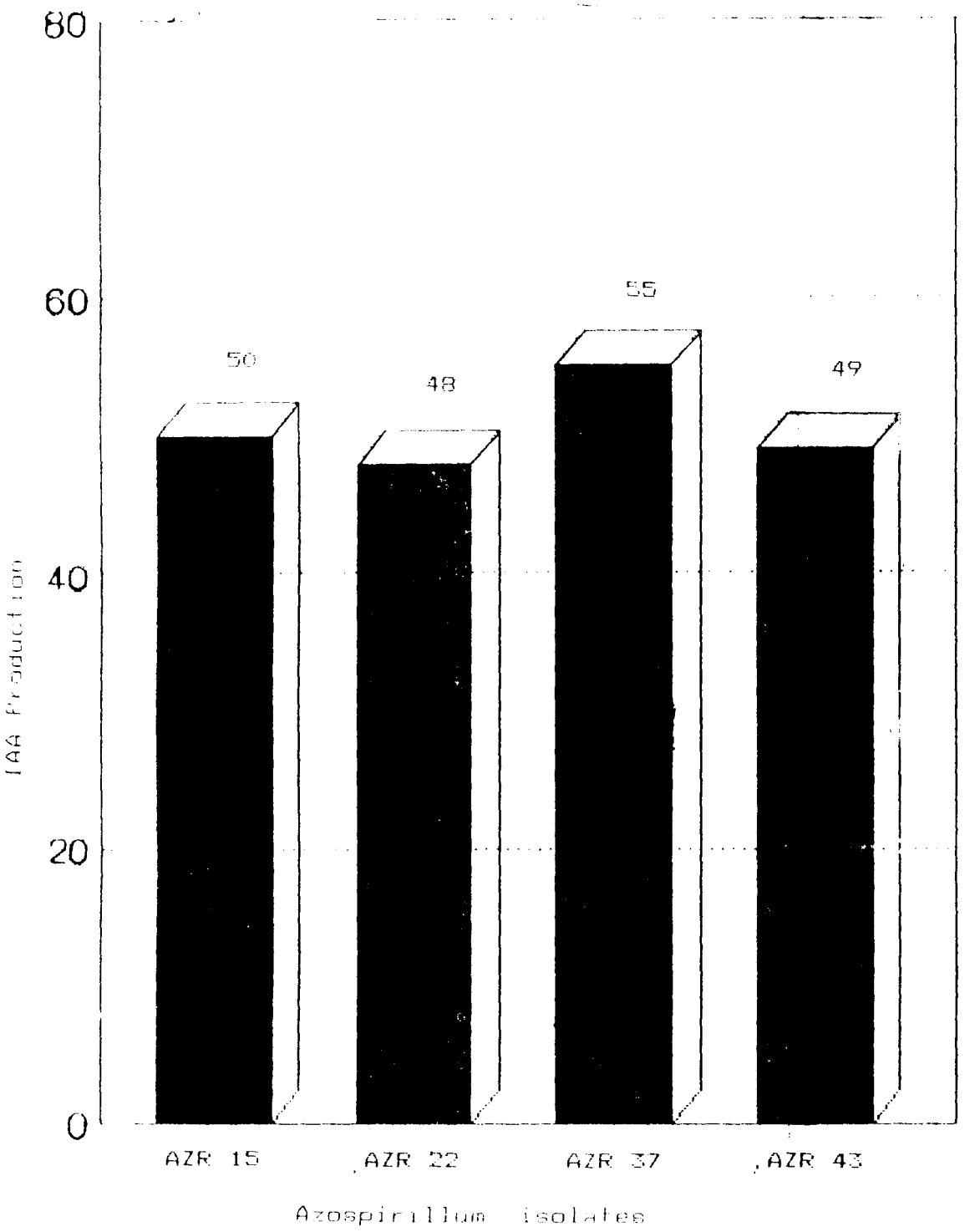


Fig.3. Indole Acetic Acid Production (µg/l) *in vitro* by the selected *Azospirillum* isolates.

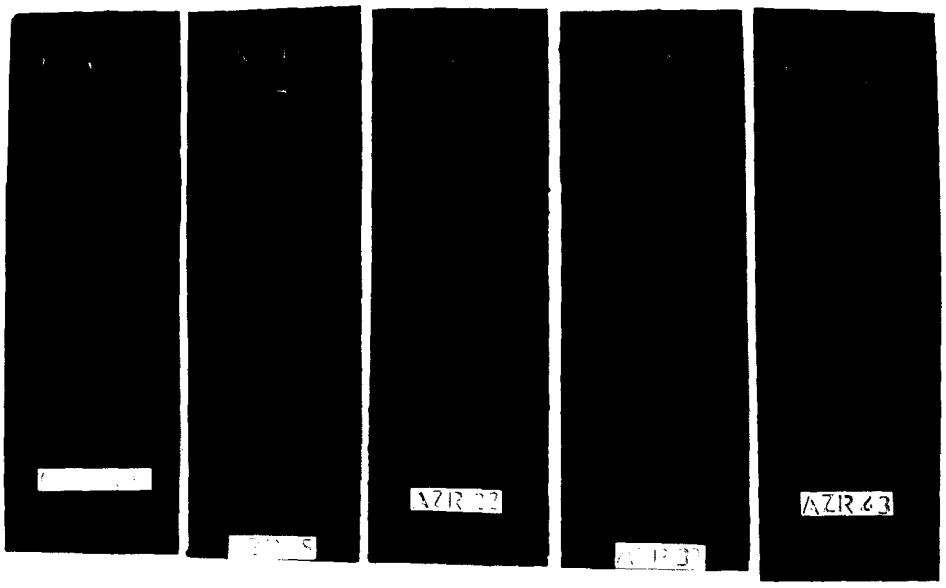


Plate.3 Effect of inoculation with selected isolates of Azospirillum on rice seedling root elongation.

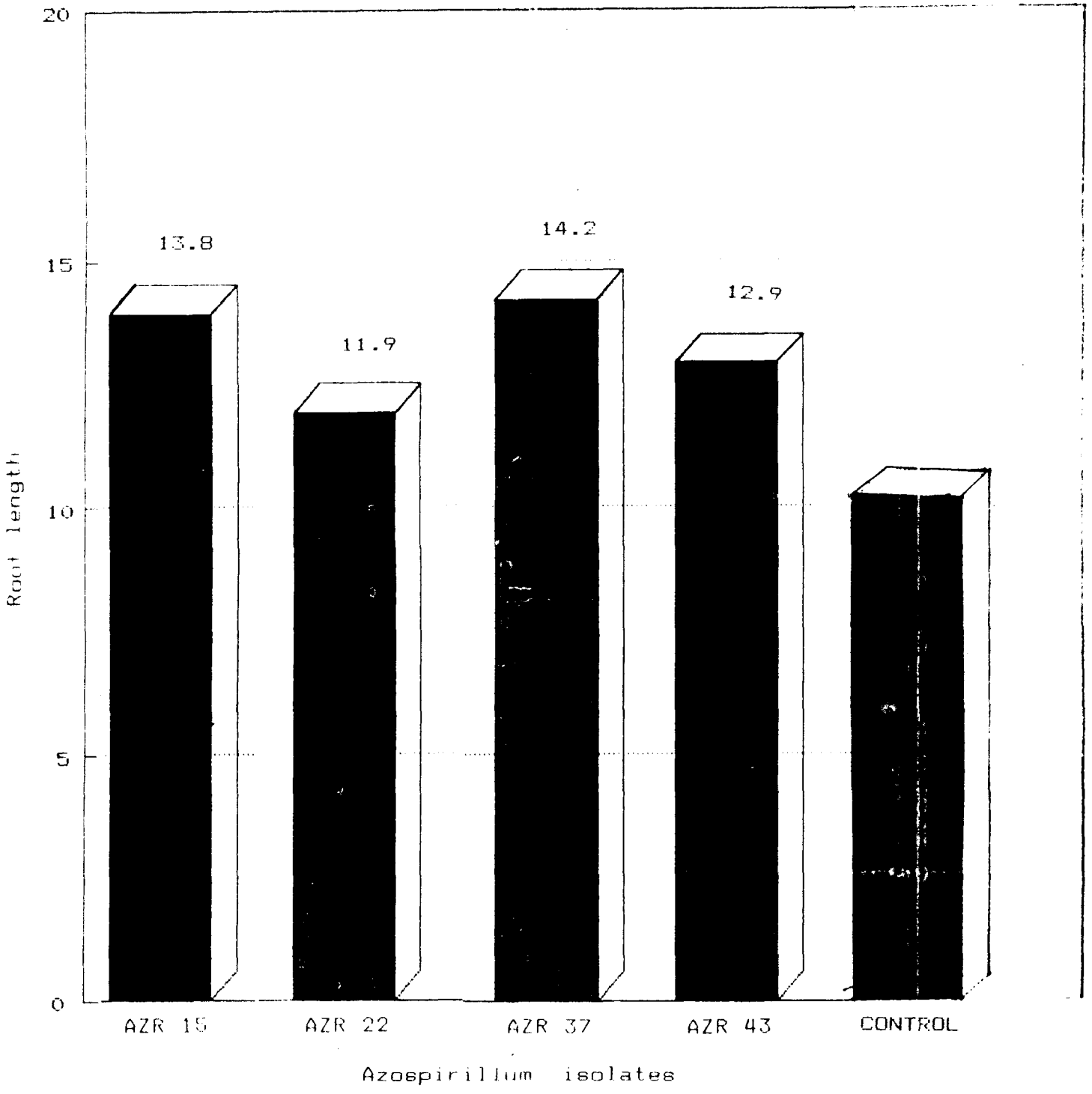


Fig.4. Root elongation (cm) in rice seedlings by the selected Azospirillum isolates.

Plate.4 Effect of inoculation with selected isolates of Azospirillum on rice (Oryza sativa.L.).

4.6.1 Height of plants on 25th day of transplanting.

All the inoculated plants gave significant increase in plant height over uninoculated control plants (Table 9). The AZR 37 inoculated plants recorded the highest value (33.30cm) followed by AZR 15 (33.03cm). Regarding the effect of chemical nitrogen, there was a general increase in plant height with increased dose of nitrogen. Application of 75% and full dose of nitrogen gave significant increase in plant height over control which recorded 33.45,33.17 and 28.82cm respectively. The interaction effect of nitrogen and inoculation was not significant.

4.6.2 Height of the plants on 50th day of transplanting

The data on plant height at 50 DAT showed that the effect of inoculation and nitrogen levels was significant (Table 10). The interaction effect was not significant. The inoculation with

Table 9 Plant height (cm) on 25 DAT as influenced by inoculation with Azopirillum isolates

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean(Isolates)
AZR 15	31.43	32.00	35.70	33.00	33.03
AZR 22	29.87	30.30	33.50	33.40	31.77
AZR 37	31.70	31.93	34.03	35.53	33.30
AZR 43	27.90	31.30	32.37	32.63	31.05
AZR 0	23.20	27.13	31.63	31.27	28.31
Mean (Nitrogen)	28.82	30.53	33.45	33.17	

C.D (isolates) - 2.4123
C.D (nitrogen) - 2.1576

Table 10 Plant Height (cm) 50 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean(Isolates)
AZR 15	55.067	53.60	55.87	54.13	55.67
AZR 22	52.90	54.83	55.23	59.93	55.73
AZR 37	56.93	57.10	60.20	60.77	58.75
AZR 43	52.23	57.80	60.57	58.23	57.21
AZR 0	51.43	56.30	57.07	55.40	55.05
Mean (nitrogen)	53.71	55.93	57.787	57.69	

C.D. (isolates) - 2.8622
C.D. (nitrogen) - 2.5600

AZR 37 was significantly superior(58.75cm) to the control (55.05cm). Even though the other isolates showed increase in plant height over the control plants the difference was not statistically significant. Zero nitrogen level gave the lowest value (28.82 cm) for plant height. The 50% level of nitrogen didnot vary significantly from the control. But 75% and 100% N levels showed significant increase over control and recorded 33.45 and 33.17 cm respectively.

4.6.3 Height of the plants on 75th day of transplanting

A perusal of data showed significant influence of Azospirillum inoculation on height of plants at 75 DAT (Table11). The AZR 22 inoculated plants recorded a maximum of 81.38cm as against 72.83cm of the control plants. The isolates 15 and 37 recorded 79.34cm and 80.47cm respectively and showed significant increase over the control (72.83cm). The application of fertilizer nitrogen increased the plant height in general. The 100% and 75% nitrogen levels influenced plant height in a positive manner and recorded a height of 80.69cm and 80.31cm respectively which were significant when compared with the control (75.53cm).

4.6.4 Height of the plants on 100th day of transplanting

The response in terms of plant height to Azospirillum inoculation was evident even at 100 DAT (Table 12). The

Table 11 Height of plants (cm) 75 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	levels of nitrogen				
	N1	N2	N3	N4	Mean (Isolate)
AZR 15	76.63	77.50	81.97	81.27	79.34
AZR 22	81.03	82.03	78.53	83.93	81.38
AZR 37	77.47	78.87	81.77	83.77	80.47
AZR 43	71.20	77.90	80.60	79.50	77.30
AZR 0	61.33	76.33	78.87	74.97	72.83
Mean (Nitrogen)	75.53	78.53	80.31	80.69	
C.D.(isolate)	-	4.87			
C.D. (nitrogen)	-	4.36			

Table 12 Height of plants (cm) 100 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean(Isolate)
AZR 15	92.60	93.10	96.80	95.83	94.58
AZR 22	91.47	96.37	94.97	101.70	96.13
AZR 37	94.70	99.17	99.73	99.80	98.35
AZR 43	94.33	98.17	99.23	98.90	97.66
AZR 0	88.73	94.97	96.13	95.07	93.73
Mean (Nitrogen)	92.37	96.35	97.37	98.26	
C.D.(isolate)	-	3.76			
C.D.(nitrogen)	-	3.36			

inoculated plant showed increase in height over the control The isolate 37 gave a maximum height of 98.35cm followed by AZR 43 (97.66cm) and AZR 22 (96.13cm). The height of control plants was 93.73. The isolates AZR 37 and AZR 43 differed significantly from the control. The increased doses of nitrogen showed an increasing effect on plant height. The 100% nitrogen level recorded maximum height of 98.26 cm which was significantly superior to the control (92.37cm).

4.6.5 Height of plants at harvest

The data showed that plant height due to inoculation was significant at harvest (Table 13). The treatments recorded almost similar plant height except AZR 37 which recorded 100.51 cm. The uninoculated control gave a height of 96.73cm. The different levels of nitrogen had significant effect on plant height at harvest. All the levels of nitrogen were significantly superior over the control. The maximum was recorded by 100% nitrogen (100.81cm) followed by 75% nitrogen (99.55cm) and control had 95.03 cm.

The effect of various Azospirillum treatments and graded nitrogen levels on tillering of rice was recorded at 25,50 and 75 DAT are presented below.

Table 13 Height of plants (cm) at harvest as influenced by inoculation with Azospirillum isolates.

Isolates	Levels of nitrogen				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	96.37	94.57	98.80	96.97	96.68
AZR 22	94.00	98.57	97.67	104.23	98.62
AZR 37	96.57	101.53	102.00	101.93	100.51
AZR 43	96.00	100.53	101.53	101.60	99.89
AZR 0	92.20	97.53	97.87	99.30	96.73
Mean (nitrogen)	95.03	98.55	99.55	100.81	
C.D. (isolate)	-	3.41			
C.D. (nitrogen)	-	3.05			

Table 14 Number of tillers 25 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	3.00	5.00	4.00	3.33	3.83
AZR 22	3.33	3.67	5.67	4.33	4.25
AZR 37	3.33	4.00	3.00	5.00	3.83
AZR 43	3.00	3.33	3.00	5.00	3.58
AZR 0	2.67	3.00	3.33	3.33	3.08
Mean (nitrogen)	3.07	3.80	3.80	4.20	
C.D. (isolate)	-	0.593			
C.D. (nitrogen)	-	0.530			

4.6.7 Number of tillers on 25 th day of transplating

The inoculation with AZR 22 showed maximum tiller production of 4.25 as against 3.08 of the control plants and AZR 15, 37 and 43 recorded 3.83, 3.83 and 3.58 respectively. These increments over control were statistically significant(Table 14). The tiller production was found to increase with increasing levels of nitrogen at 25 DAT. The 100% level gave maximum tiller production of 4.20 followed by 50% (3.80). All these results were significant statistically.

4.6.8 Number of tillers on 50 th day of transplanting.

Obeservations on tiller production at 50 DAT (Table 15) indicated a consistent increase in tillering due to Azosprilium inoculation AZR 37 gave maximum value (6.58) followed by AZR 22 (6.33) and AZR 15 (6.17). The control plants recorded a tiller production of 5.00. The application of the different nitrogen levels retained the general trend as oberseved at 25 DAT. The influence of nitrogen application at 75% and 100% levels were significantly higher than control (5.33).

Table 15 Number of tillers 50 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	5.67	6.67	6.00	6.33	6.17
AZR 22	5.33	6.00	7.67	6.33	6.33
AZR 37	6.33	6.33	6.67	7.00	6.58
AZR 43	5.33	4.67	5.67	6.67	5.58
AZR 0	4.00	5.00	5.33	5.67	5.00
Mean (nitrogen)	5.33	5.73	6.27	6.40	

C.D. (isolate) - 0.690
C.D. (nitrogen) - 0.617

Table 16 Number of tillers 75 DAT as influenced by inoculation with Azospirillum isolates.

Isolate	Levels of nitrogen				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	9.33	8.67	9.33	8.33	8.92
AZR 22	8.00	8.33	9.00	9.00	8.58
AZR 37	8.33	9.33	9.33	9.33	9.08
AZR 43	9.00	8.67	8.33	9.00	8.75
AZR 0	8.00	8.33	8.33	9.00	8.42
Mean (nitrogen)	8.53	8.67	8.87	8.93	

C.D. (isolate) - 0.698
C.D. (nitrogen) - 0.624

4.6.9 Number of tillers on 75 th day of transplanting

A close scrutiny of the data on tiller production 75 DAT (Table 16) showed no significant effect of inoculation. Isolate 37 gave the maximum value of 9.08. The control recorded a minimum value of 8.42. There was not much difference among the effects of nitrogen levels on tiller production. However 100% nitrogen showed a marginal increase (8.93) over the control (8.53).There was no significant interaction effect between the Azospirillum and nitrogen levels on tiller production at any growth stage of the plant.

4.6.10 Productive tillers

A perusal of data on productive tillers (Table 17) revealed that AZR 37 and 15 gave maximum value of 7.17 and 6.92 respectively, and were significantly superior to control (6.08). The application of chemical nitrogen influenced the productive tillers at all graded levels and were statistically significant.There was no significant interaction between Azospirillum and nitrogen application on productive tillers.

4.6.11 Grain yield

The effect of inoculation with Azospirillum and graded levels of fertilizer nitrogen on the grain yield of rice was

Table 17 Effect of inoculation with Azospirillum isolates on the number of productive tillers.

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	6.67	7.00	7.37	6.67	6.92
AZR 22	6.00	6.33	6.67	7.00	6.50
AZR 37	6.00	7.67	7.33	7.67	7.17
AZR 43	5.67	6.67	6.67	6.33	6.33
AZR 0	5.67	5.33	6.67	6.67	6.08
Mean (nitrogen)	6.00	6.60	6.93	6.87	

C.D. (isolate) - 0.6479
C.D. (nitrogen) - 0.5795

recorded at harvest and is presented in the table 18. AZR 15 recorded the highest grain yield of 11.042 g. All the isolates inoculated showed a positive influence on the grain yield . The control plants recorded the lowest yeild of 9.823g. The full dose of nitrogen along with AZR 37 gave a maximum of 11.73 g as against 10.75 g recorded for full dose of nitrogen alone . Azospirillum isolates AZR 15, 22 and 37 along with 75% N recorded an yeild of 11.33g, 11.07g and 11.33g respectively .The interaction effect of Azospirillum and nitrogen levels was not significant.

4.6.12 Thousand grain weight

The influence of inoculation with Azospirillum and different nitrogen levels on 1000 grain weight is given in the table 19 . AZR 37 recorded a maximum of 26.82 g followed by AZR 15 (26.33g), AZR 22(26.08g), AZR 43(24.72g) and control (24.77g). All the treatments except AZR 43 showed significant influence on 1000 grain weight but they did not vary much among themselves. The nitrogen levels also influenced 1000 grain weight positively. A maximum of 26.41g was recorded by 100% nitrogen followed by 26.18g by 75% nitrogen. Nitrogen at 50% nitrogen had no significant influence when compared to control. The interaction effect was not significant.

Table 18 Effect of inoculation with Azospirillum on grain yield in rice.(g/pot)

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (Isolate)
AZR 15	10.62	10.72	11.33	11.49	11.042
AZR 22	9.83	10.03	11.07	11.59	10.636
AZR 37	10.07	10.08	11.37	11.73	10.788
AZR 43	9.17	10.23	10.55	10.62	10.142
AZR 0	8.77	9.40	10.37	10.75	9.823
Mean (nitrogen)	9.69	10.09	10.92	11.23	

C.D. (isolates) - 0.8690

C.D. (nitrogen) - 0.7772

Table 19 Effect of inoculation with Azospirillum isolates on 1000 grain weight (g)

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean(isolate)
AZR 15	25.23	25.63	26.67	27.77	26.33
AZR 22	24.90	25.66	27.00	26.67	26.08
AZR 37	26.70	26.50	27.17	26.90	26.82
AZR 43	24.30	24.60	24.57	25.40	24.72
AZR 0	24.50	24.60	24.57	25.40	24.77
Mean (nitrogen)	25.14	25.46	26.18	26.41	

C.D (isolate) - 0.7880
C.D (nitrogen) _ 0.7048

4.6.13 Straw yield

The data observed for sun dried straw is presented in the table 20 . The isolate 37 recorded the highest straw yield of 15.20g which was significantly higher than that of control (13.56). The rest of the values did not vary significantly from control. The nitrogen levels influenced straw yield positively. The 100% level gave maximum yield of 15.14g followed by 75% (14.66g). The control recorded a minimum of 13.54g. The influence of 75% and 100% nitrogen level was significant whereas that of 50% nitrogen was not. There was no interaction effect.

4.6.14 Root weight

The root weight of the plant was recorded at the time of harvest and the data is presented in table 21. The maximum root weight was recorded by isolate AZR 15 inoculated plants which recorded 1.99g as against 1.60g observed for control. However the increase in root weight due to inoculation and nitrogen application was not significant.

4.6.15 Root length

The effect of Azospirillum inoculation and nitrogen applicion on root length was studied and presented in table 22. The maximum root length of 12.89 cm was recorded by isolate AZR15

15. The control recorded 12.18 cm. The isolates AZR 15, 22 and 37 influenced the root length significantly. There was not much variation among the influence of isolates on root length. The nitrogen levels did not show significant influence on root length except in the case of 100% N which showed a maximum root length of 12.77cm. The interaction effect on the root length was not significant.

4.7 Chemical analysis of plant

The analysis of data on total nitrogen content of plants (Table 23)indicated non significant effect due to inoculation. However the influence of fertilizer nitrogen was significant. Although there was no significant difference in nitrogen uptake, AZR 37 gave the maximum value for nitrogen content.(344.08mg) followed by AZR 15(337.00mg) and control(309.25mg). This accounts to 11.26 and 8.97% increase in total nitrogen uptake over control plants. The increase in nitrogen application influenced nitrogen content of plants. The 100% nitrogen and 75% nitrogen levels recorded values of 357.33 and 345.27mg respectively which were significant increases over the control (304.80mg).

The data on percent protein content of the grains are presented in table 24 . The protein content did not show any

Table 20 Effect of inoculation with Azospirillum isolates on straw yield (g/pot) of rice.

Isolate	Nitrogren levels				
	N1	N2	N3	N4	Mean(isolate)
AZR 15	13.41	13.92	14.92	15.18	14.50
AZR 22	13.62	13.93	14.42	15.01	14.25
AZR 37	15.19	14.42	15.50	15.69	15.20
AZR 43	13.11	13.10	14.90	14.97	14.01
AZR 0	12.39	13.44	13.45	14.97	13.56
Mean (nitrogen)	13.54	13.76	14.66	15.14	

C.D (isolate) - 1.0155
C.D (nitrogen - 0.9083

Table 21 Root weight (g/pot) of rice plants as influenced by inoculation with Azospirillum isolate

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	1.51	1.74	1.76	1.86	1.72
AZR 22	1.46	1.51	1.57	1.64	1.54
AZR 37	1.70	1.77	1.87	1.87	1.80
AZR 43	1.61	1.67	1.75	1.80	1.99
AZR 0	1.50	1.54	1.59	1.76	1.60
Mean (nitrogen)	1.56	1.65	1.71	1.78	

C.D (isolate) - 9.99
C.D (nitrogen) - 8.94

Table 22 Root length (cm) of rice plants as influenced by inoculation with Azospirillum isolates.

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	12.93	12.86	12.90	12.93	12.89
AZR 22	12.43	12.47	12.90	13.07	12.72
AZR 37	12.37	12.90	12.90	13.00	12.79
AZR 43	11.97	11.87	11.97	12.37	12.04
AZR 0	11.93	12.03	12.30	12.40	12.18
Mean (nitrogen)	12.33	12.41	12.59	12.77	

C.D. (isolate) - 0.4719
C.D. (nitrogen) - 0.4221

Table 23 Nitrogen content (mg) in rice plants as influenced by inoculation with Azospirillum isolates.

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	310.67	324.33	355.00	358.00	337.00
AZR 22	313.33	312.33	340.33	357.33	330.83
AZR 37	331.33	309.00	361.33	374.67	344.03
AZR 43	300.00	315.00	351.00	350.33	329.08
AZR 0	268.68	303.33	318.67	346.33	309.25
Mean (nitrogen)	304.80	312.80	345.27	357.33	

C.D (isolates) 24.4189
C.D (nitrogen) 21.8409

Table 24 Protein content (%) of grains as influenced by inoculation with Azospirillum isolates.

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	8.04	8.23	8.38	8.08	8.18
AZR 22	8.42	8.04	8.23	8.19	8.22
AZR 37	8.38	7.75	8.23	8.38	8.13
AZR 43	8.38	8.04	8.23	8.42	8.27
AZR 0	7.75	8.37	7.89	8.04	8.02
Mean (nitrogen)	8.19	8.09	8.19	8.22	

C.D. (isolate) - 0.3115
C.D. (nitrogen) - 0.2786

Table 25 Total nitrogen content of soil (mg/pot) after the experiment as influenced by inoculation with Azospirillum isolates and nitrogen application.

Isolate	Nitrogen levels				
	N1	N2	N3	N4	Mean (isolate)
AZR 15	3466.67	3500.00	3566.67	3600.00	3533.33
AZR 22	3450.00	3533.33	3566.67	3600.00	3537.50
AZR 37	3433.33	3533.33	3566.67	3600.00	3533.33
AZR 43	3466.67	3533.33	3566.67	3633.33	3550.00
AZR 0	3466.67	3500.00	3566.67	3600.00	3533.33
Mean (nitrogen)	3456.67	3520.00	3566.66	3606.67	

C.D (isolate) - 43.5587
C.D.(nitrogen) - 38.9600

significant difference on inoculation with Azospirillum. However the inoculated plants showed a slight increase in the protein content over the control. Similarly the increase in the nitrogen level also did not influence the protein content significantly.

4.8 Analysis of nitrogen balance in the soil

The analysis of nitrogen balance was made after the harvest of the crop (Table 25) and the nitrogen gain in the system was computed. The data obtained is presented in the table 26.

There was a general increase in the value of nitrogen balance for all the inoculation treatments. Fertilizer nitrogen application further improved nitrogen balance. In the unfertilized treatments AZR 15, 22, 37 and 43 recorded values of 177.165 and 167mg respectively against 135mg recorded for the control. This accounts to a gain of 42, 28, 30 and 31mg of nitrogen per pot due to inoculation of respective Azospirillum isolates. This constitute 31, 21, 22 and 23 kg of fixed nitrogen per hectare. The nitrogen balanced values were found to increase with higher doses of nitrogen application. Application of nitrogen up to 75% did not considerably affect nitrogen fixation. But 100% nitrogen generally showed an inhibitory effect on biological nitrogen fixation.

Table 26 Nitrogen balance as influenced by inoculation with Azospirillum isolates and nitrogen application

Isolates	Nitrogen added mg/pot	Total nitrogen mg/pot	Soil nitrogen after experiment	Plant nitrogen mg	Total nitrogen recovered	Nitrogen balance mg/pot	Nitrogen fixation mg/pot	Nitrogen fixed kg/ha
AZR15N1	0	3600	3466.66	310.67	3777.33	177.33	41.99	30.79
AZR15N2	90	3690	3500.00	324.33	3824.33	134.33	20.99	15.39
AZR15N3	135	3735	3566.67	355.00	3921.67	186.67	36.33	26.64
AZR15N4	180	3780	3600.00	358.00	3958.00	178.00	11.63	8.53
AZR22N1	0	3600	3450.00	313.33	3763.33	163.33	27.99	20.53
AZR22N2	90	3690	3533.33	312.33	3845.66	155.66	42.32	31.34
AZR22N3	135	3735	3566.67	340.43	3907.00	172.00	21.66	15.84
AZR22N4	180	3780	3600.00	357.33	3957.33	177.00	10.63	8.04
AZR37N1	0	3600	3433.33	331.33	3764.66	164.66	29.66	21.75
AZR37N2	90	3690	3533.33	309.00	3842.33	152.33	38.96	28.57
AZR37N3	135	3735	3566.67	361.33	3928.00	193.00	42.66	31.28
AZR37N4	180	3780	3600.00	374.67	3974.67	194.67	28.3	20.15
AZR43N1	0	3600	3466.67	300.60	3766.67	166.67	31.33	22.98
AZR43N2	90	3690	3533.33	315.00	3848.33	158.33	44.99	32.99
AZR43N3	135	3735	3566.67	351.00	3917.67	182.67	32.33	23.47
AZR43N4	180	3780	3633.33	350.33	3983.66	203.66	37.21	27.35
AZR0N1	0	3600	3466.67	268.67	3735.34	135.34		
AZR0N2	90	3690	3500.00	303.33	3803.33	113.34		
AZR0N3	135	3735	3566.67	318.67	3885.34	150.34		
AZR0N4	180	3780	3600.00	346.37	3946.37	166.37		

4.9 Analysis of genetic basis of salt tolerance.

The curing of plasmids to study the genetic base of salt tolerance was done using Acridine orange and sodium dodecyl sulphate and the data is presented in the tables 27 and 28 .

4.9.1 Acridine orange curing

The acridine orange curing of plasmids with 10 ppm and 20 ppm concentrations resulted in a percent curing of 8.1 and 6.2 of AZR 8 respectievely.In AZR 29 there was 2.0 and 4.5 percent curing of plasmids at 10 and 20 ppm respectievely (Table 27)

4.9.2 Sodium dodecyl sulphate curing

The treatment with SDS (1.0%) recorded 5.1 and 6.6 percent curing of AZR 8 and AZR 29 respectively. (Table 27)

Table 27 Effect of plasmid curing with acridine orange on salt tolerance of *Azospirillum* isolates.

Isolate	Acridine orange concentration (ppm)	Curing percent
AZR 8	0	-
	5	0
	10	8.1
	20	6.2
AZR 29	0	-
	5	2.0
	10	4.5
	20	0

Table 28 Effect of plasmid curing with Sodium dodecyl sulphate (SDS) on salt tolerance of *Azospirillum* isolates

Isolate	SDS concentration	Curing percent
AZR 8	1%	5.1
AZR 29	1%	6.6

CHAPTER V

DISCUSSION

Isolation and characterisation of *Azospirillum*

In the present study an attempt was made to isolate *Azospirillum* of rice plants from different locations in Kuttanad and to develop an inoculant culture for the acid sulphate soils of the region.

The chloramine T (1%) used for surface sterilization of root bits reduced surface contaminants. The semi solid medium encouraged the growth of *Azospirillum*, probably by providing a microaerophilic condition and there by the right oxygen concentration. (Okon *et al.*, 1980). But contamination with some bacteria and proactinomycetes were frequently observed. This may be due to the growth of other microaerophilic organisms along with *Azospirillum* (Mishustin and Shilinikova, 1969; Raju *et al.*, 1972; Lekshmi Kumari *et al.*, 1976). The growth of *Azospirillum* occurred as a white undulating pellicle below the meniscus. (Burlow and Dobereiner, 1975; Lekshmi Kumari et al, 1976; Burris *et al.*, 1977). The purified isolates on transfer to semisolid medium showed the formation of characteristic white undulating pellicle and the presence of *Azospirillum* was confirmed. Lekshmi Kumari *et al.*, 1976). The purified isolates were then subjected to morphological and physiological studies.

The isolates were Gram negative motile and contained polybeta hydroxy butyrate granules. They were short to medium sized and slightly curved rod shaped. Polymorphism was noted in most of the cultures. The morphological observation of the isolates agreed with the descriptions given by Dobereiner and Day (1976) and Okon et al., (1976 a,b) for Azospirillum. The isolates were then subjected to detailed physiological and biochemical studies for species level identification. Among the fifty isolates eighteen of used glucose and all utilised fructose as sole carbon source (Table 2) Among those which failed to use glucose failed to use sucrose and acidify peptone based glucose medium (Table 5) and showed growth on medium without biotin (Table 3). The observations were in accordance with the description of Azospirillum brasilense under the Group I of Tarrand et al.(1978). The isolates which exhibited good growth in glucose grew in sucrose also and acidified peptone based glucose medium. They showed the requirement of biotin for growth. Based on these observations it is inferred that the isolates belong to Azospirillum lipoferum (Tarrand et al.,1978). However, some isolates showed variable behaviour

Thirteen isolates, among the total of 50 grew in NaCl supplemented medium and were concluded to be salt dependent. All the isolates showed high salt tolerance up to 3.0% NaCl. (Table 6). Isolates 2,6,8,13 and 29 tolerated up to 4.0% NaCl

concentration. This extremely high salt tolerance might be due to the peculiar characteristic of the habitat from where they were isolated. The soil of the region is of acid sulphate type having high acidity and is prone to frequent salt water inundation. Similarly all the isolates grew well at a pH of 5.0 and 19 showed growth even at pH 4.3. Six isolates recorded growth at pH 4.0 (Table 7). This pH tolerance might be the physiological adaptation of the organism to survive the extreme low pH of the habitat. In the acid sulphate soils the pH may go even up to 3.2 to 3.0.

The estimation of nitrogen fixation *in vitro* by the microKjeldhal method showed wide variations in the ability of isolates to fix nitrogen (Table 8). Many authors have reported that nitrogen fixation *in vitro* by *Azospirillum* is highly variable, perhaps this is due to the variation in their inherent capacity to fix nitrogen (Day and Dobereiner, 1976; Okon *et al.*, 1976 a; 1976 b; Nelson and Knowles, 1978). The isolate 15 gave maximum nitrogen fixation of 21.28mg/g malate. The lowest fixation of 11.20mg/g malate was recorded by many isolates. The nitrogen fixation by the isolates were in the range observed by many workers. (Okon *et al.*, 1976 and Nelson and Knowles, 1978). Many workers have opined that the major effects of *Azospirillum* inoculation on host plants are its contribution

through nitrogen fixation and hormonal effect on root proliferation and thereby better plant growth (Kapulnik et al 1985 a,b; Kolb and Martin, 1985; Levamony and Bashan, 1989).

The studies on IAA production also showed wide variation by the isolates as in the case of nitrogen fixation (Table 8). The production of IAA by the isolates ranged between 24 µg l^{-1} and 55 µg l^{-1}. Such variation in the production of IAA by Azospirillum strains was reported by Tien et al., 1979. The IAA production by the bacteria has direct influence on the root growth and elongation (Kapulnik, 1985). It should be noted that in the present investigation isolates which produced more IAA showed better root growth in the root elongation studies (Table 8). The isolates AZR 15 and 37 producted 50 and 55 µg/l IAA respectively and the corresponding root lengths were 13.8 and 14.2 cm .

The isolates for further studies were selected based on nitrogen fixation, IAA production and root elongation. Since the Kuttanad tract is highly acidic and prone to salt water inundation, the pH and salt tolerance of the isolates were also considered in the selection of isolates. Thus, the isolates AZR 15, 22, 37 and 43 which showed satisfactory tolerance to low pH and high salinity were chosen for the pot culture trials. The

pot culture trials were conducted in green house to study the effect of inoculation of the selected isolates on nitrogen fixation, plant growth characteristics and yield at graded levels of fertilizer nitrogen.

The analysis of the data on plant height recorded at different growth stages revealed that the inoculated plant showed significant increase in plant height during the early growth stages (25 and 50 DAT) and was similar to the observations made by Karthikeyan, 1981, Watanabe and Lin, 1984, Gopalaswamy and Vidyasekharan, 1987. The isolate 37 gave the highest value for plant height. The early growth stimulation could be due the effect of hormones produced by the Azospirillum and/or due to nitrogen fixation. During later stages the declining carbon reserves and increasing ammonium levels restricted the nitrogenase activity (Van Berkum and Sloger, 1983) and the temporary increase in nitrogenase activity becomes insignificant as the rice plants grew older (Rao and Rao,1983, Watanabe and Lin, 1984). The reduction in the nitrogen fixation and hormone production in the later stages by the inoculated plants might be the reasons why the control plants picked up the growth during the later stages. Perhaps the reduction in nitrogen fixation and hormone production may be the reasons for the reduced influence of inoculation on the rice plant height at later stages. The

increase in plant height due to inoculation may be attributed to the production of Gibberillins by A. lipoferum (Bottini et al,1989). Similarly the increased tillering of the inoculated plants was more pronounced during the early stages of growth (50 DAT). Similar observations were made by Watanabe and Lin (1984): Goplalaswamy and Vidyasekharan (1987). The increased nitrogen fixation and hormone production in the early stages might have positively influenced tiller production also. The present study showed that increased levels of fertilizer nitrogen increased plant height and tiller production. This emphasises the earlier view that the increased nitrogen fixation during the earlier stages might have influenced the increased tiller production at early stages. According to Christiansen-Weniger (1988), the nitrogen fixing capability and hormone production are directly related. In the present study it is evident from higher nitrogen fixation of the isolates which recorded higher amounts of hormone production.

A perusal of data on productive tillers (Table 17) showed that inoculation had significant influence. From the economic point of view this aspect is very important as this is one of the major factors that determines the grain yield. The increase in productive tiller number due to inoculation was noticed by Baschan, 1986a, Bouton and Zuberer, 1979 . The isolate AZR 15

and AZR 37 gave highest values for productive tillers. The beneficial effects on root parameters on inoculation might have influenced the mineral uptake by plants (Barton et al., 1986: Jain and Patriquin,1985; Kapulnik at al., 1985b: Morgenatern and Okon,1987). The accumulation of these minerals in plant body might have been utilised during the reproductive period for the formation of more productive tillers. The increased production of productive tillers by AZR 15 and AZR 37 supports the fact that root development is improved by hormone production of the isolates,as is evident from their higher hormone production in vitro.

The data on root elongation and root weight showed that the inoculated plants gave higher values over control. The inoculation effect on roots is clearly evident from the increased root length shown by inoculated plants. This is due to the increased elongation of the root elongation zone (Kalpulnik et al 1985,a 1985 b ; Kolb and Martin, 1985; Levemony and Bashan, 1989). The inoculation with Azospirillum increased the number of lateral roots, root hairs and total root volume and dry weight. The cell division in the meristems which lead to elongation of roots and proliferation might be the reasons behind this(Dewan and Subba Rao, 1979). Perhaps some of the secondary metabolities

of Azospirillum might have influenced the physiological process of root growth. (Levamony and Bashan, 1989). Such increase in root proliferation and root length on Azospirillum inoculation was observed (Tien et al, 1979: Kolb and Martin, 1985: Barber: et al 1986: Morgensten and Okon 1987: Fallik et al 1988). The efficiency of hormone production in vitro by the isolates AZR 15 and 37 influenced the root elongation of rice in the pot studies also.

The sundried straw yield at harvest (Table 20) was studied and the inoculated plants gave higher straw yields over the control. As stated earlier the increased mineral uptake and accumulation due to positive effects on root parameters might have resulted in increased biomass production. This is evident from the higher number of tillers produced by inoculated plants. Similar increase in straw yields due to inoculation was reported by earlier workers (Rao et al 1983., Prasad and Singh 1984. Balasubramanium and Kumar 1987 and Gopalaswamy, 1992). The maximum straw yield was given by AZR 37.

The inoculation with Azospirillum influenced the grain yield in rice. All the inoculated plants gave higher yields than the control plants. The increase in yield due to inoculation with Azospirillum is attributed to the sum total of the benefical effects of inoculation on root parameters like root length,

proliferation, volume, dry weight and surface area. This helps in increased nutrient uptake and biomass production (Barton et al., 1986; Jain and Patriquin, 1984; Sariq et al., 1988). In the present study also it could be seen that there was no much difference in the root length and root dry weight between the isolates 15 and 37 and these isolates recorded almost similar grain yields of 11.04 and 10.79g respectively.

The full dose of nitrogen along with AZR 37 gave a maximum yield of 11.73g (Table 18). The application of 75% N along with AZR 15 and AZR 37 inoculation resulted in a grain yield of 11.33 and 11.37g respectively. Thus, it is concluded that the application of 25% N can be saved by inoculation with these cultures without sacrificing total grain yield.

The nitrogen content of plants (Table 23) increased on inoculation with Azospirillum. AZR 37 and AZR 15 showed an increase of approximately 11 and 9 percent over the control. However the increase was not statistically significant. A similar trend was noticed in the case of protein content of grains also. The inoculated plants showed a marginal increase in the protein content of grains. Such observations were also reported by Watanabe and Lin, 1984. The increase in nitrogen content of plants on inoculation may be attributed to the

enhanced uptake of NO_3 and NH_4 and nitrogen fixation by the inoculated plants (Barton et al., 1986; Jain and Patriquin, 1984). This might have caused an increased accumulation of these minerals in the stem and leaves. In the present study AZR 15 and AZR 37 showed better performance in all the root parameters and biological nitrogen fixation and this might have influenced the total mineral uptake and hence the increased N content of plants inoculated with these isolates.

The total nitrogen content of the soil after the experiment (Table 25) did not show any significant change among the treatments. But the nitrogen balance studies (Table 26) showed that the inoculated plants gained more nitrogen than the uninoculated plants. This indicated that the treatments did not influence the nitrogen uptake from soil source. However there was an increase in the left over nitrogen of soil with increased fertilizer nitrogen application. The total N recovered (Initial soil N + plant N) was consistently higher in the inoculated plants. This indicates the nitrogen contribution in the system due to biological nitrogen fixation by Azospirillum. The analysis of the values computed for N balance showed clear nitrogen gains due to the inoculation. A nitrogen balance of 177.33, 163.33, 164.66 and 166.67 nmg per pot was recorded for

isolates AZR 15, 22 and 43 respectively as against 135.34 observed for the control at 0 level nitrogen. This accounts for 31.02, 20.07, 21.66, 23.15 per cent increase in nitrogen balance due to inoculation. Nitrogen application showed a general trend of increasing N balance with increasing dosages. The higher N balance for the inoculated plants confirms the nitrogen contribution due to biological nitrogen fixation by Azosopirillum. The value derived for total biological nitrogen fixation by various isolates showed that inoculation with Azospirillum can contribute about 20-30Kg/ha when no nitrogen fertilizer was applied. Chemical nitrogen application up to 75% level did not generally affect the biological nitrogen fixation. However 100% application showed a deleterious effect on biological nitrogen fixation. However, it should be emphasised that Azospirillum inoculation along with 75% nitrogen application is economical and environment friendly rather than 100% nitrogen application.

Curing of plasmids with acridine orange and sodium dodecyl sulphate resulted in the loss of salt tolerance characteristic in the replica plate at a very low frequency. This indicated that the salinity tolerance obaserved in the isolates was plasmid borne. Such plasmid borne nature of salinity tolerance in Azospirillum has been

noticed (Balasubramoniam, 1987). Hence it could be possible to make use of this genome in genetic engineering work for inducting salt tolerance in desirable organism. However the frequency of cured colonies noticed was very low, it is to be confirmed with more sophisticated techniques.

CHAPTER VI

SUMMARY

The isolation of Azospirillum was made from 25 different locations in Kuttanad, Kerala with an aim to develop acid and salt tolerant inoculant cultures suited for the highly acidic acid sulphate soils of the locality. A total of 50 isolates were obtained. Morphological studies showed that they were Gram negative,motile,slightly curved rod shaped and contained poly beta hydroxy granules. Based on morphological,physiological and biochemical studies the isolates were identified as A. lipoferum and A brasilense. Among the 50 isolates 13 were salt dependent. All the isolates showed high salt tolerance upto 2.0% NaCl AZR.2,6,8,13 and 29 tolerated up to 4.0% NaCl. All the isolates grew well at pH 5.0,17 at pH 4.3 and 5 showed growth at pH 4.0

The nitrogen fixing capacity of the isolates showed wide variations. AZR 15 recorded the maximum of 21.28mg N/g malate. The lowest rate of 11.20mg N/g malate was recorded by many isolates. The production of IAA by the isolates ranged between 26 $\mu g\ l^{-1}$ and 55 $\mu g\ l^{-1}$. The root elongation study was conducted to study the effect of inoculation on root length. The isolate AZR 37 gave maximum root length followed by AZR 15. These isolates produced higher amounts of IAA in vitro and this may be the reason for the better root elongation of rice seedlings.

The isolates for pot culture trials were selected based on nitrogen fixation, IAA production and root elongation. The pH and salt tolerance of the isolates were also considered since the Kuttanad tract is highly acidic and prone to salt water inundation. The isolates AZR 15, 22, 37 and 43 were chosen for the trial. The treatments were combinations of isolate with the graded levels of fertilizer nitrogen (N1-O nitrogen, N2 - 50%,N3-75% and N4-100% of Package of practices recommendations) Other management practices were as per the recommendations.

The effect of inoculation was most evident during the early stage of growth (25 and 50 DAT). Eventhough this was less pronounced during the later stages the difference was clearly evident. Similarly the inoculated plants produced more number of productive tillers. AZR 15 and 37 gave the best results. All Azorpirillum inoculated plants gave higher yields than the control plants. The full dose of nitrogen with AZR 37 produced maximum yield of 11.71g. The inoculation with AZR 15 and AZR 37 along with 75% N application resulted in grain yield of 11.23g and 11.30g respectively. The straw yield on inoculation was greater than control. But only isolate AZR 37 gave statistically significant result.

The nitrogen content of plants increased as a result of inoculation with Azospirillum. Similarly protein content of grains also increased on inoculation. However these two observations were not statistically significant. The analysis of nitrogen balance showed nitrogen contribution in the system due to biological nitrogen fixation by Azozpirillum. The values derived for total nitrogen fixation by various isolates showed that the inoculation with Azospirillum can contribute about 20-30kg/ha when no nitrogen fertilizer was applied. The application of 75% level of nitrogen did not affect biological nitrogen fixation and was economical. A saving of 25% nitrogen can be made by inoculation with the cultures with out affecting biological nitrogen fixation and sacrificing grain yield. However it needs to be tested in the fields of Kuttanad.

The acridine orange curing of plasmids conducted to know the genetic basis of salt tolerance, showed that the character is plasmid borne. Hence it could be possible to make use of the genome in genetic engineering work for inducting salt tolerance in desirable organisms.

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Appendix-1

Composition of media used.

(a) Nitrogen Free Bromothymol blue (NFb) medium

(Baldani and Dobereiner,1980)

Malic acid	5g
$K_2H\ PO_4$	0.5g
$Mg\ SO_4\ 7H_2O$	0.2g
NaCl	0.1g
$CaCl_2$	0.02g
Trace element solution	2ml
Alcoholic solution of bromothymol blue (5%)	2ml
Fe EDTA (1.64% W/V aqueous)	4ml
Vitamin solution	4ml
KOH	4g
Agar	1.75g
Distilled water	1000ml
pH	6.8

Trace element Solution is prepared as follows.

$Na\ MoO_4\ 2H_2O$	200mg
$MnSO_4\ H_2O$	235mg
$H_3\ BO_3$	280mg
$Cu\ SO_4\ 5H_2O$	8mg
$Zn\ SO_4\ 7H_2O$	24mg
Distilled Water	200ml

Vitamin solution is prepared as follows.

Biotin	10mg
Pyridoxin	20mg
Distilled water	100ml

(b) Potato infusion agar

(Baldani and Dabereiner,1980)

Potatoes	200g
malic acid	2.5g
KOH	2.0g
Sucrose	2.5g
Vitamin Solution	1.0ml
Agar	15g

Washed potatoes were boiled for 30 minutes and the solution was filtered. Malic acid was dissolved in 50ml H_2O, adding two drops of bromothymol blue (0.5% solution of ethanol) and pH adjusted to 7.0 with KOH (green colour). Sucrose, vitamins and agar were added to the potato filtrate and made up to 1000ml.

ABSTRACT

Azospirillum was isolated from the rice roots of twenty five different locations of Kuttanad, Kerala in order to develop acid and salt tolerant inoculant cultures suited for the locality. The isolates were identified as A lipoferum and A brasilense. All the isolates showed good salt tolerance up to 2% NaCl concentration and good acid tolerance up to pH 5 .0 and. some isolates tolerated up to pH 4.0. The nitrogen fixing capacity in vitro by the isolates ranged between 11.20 and 2.28mg g malate and IAA production between 24 µg l-1 and 55 µg l-1. Four isolates of them were selected for pot culture trials based on efficiency of nitrogen fixation, IAA production, root elongation and acid and salt tolerance. The inoculated plants showed increased height, number of productive tillers and beneficial influence on root parameters. All these reflected in the final grain yield and AZR 15 gave highest yield folowed by AZR 37. A saving of 25% nitrogen can be made by inoculation with these cultures in rice.

The acridine orange curing of plasmids conducted to know the genetic basis of salt tolerance showed that the character is plasmid borne.