Evaluation of a Biofertilizer, Azospirillum

From Acid Soils of Kerala State

By

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Introduction

INTRODUCTION

In the recent years, with increasing cost of fertilizers (Hardy et al, 1977) man has started searching for newer technologies in agriculture where by the cost of production can be reduced and the fertility of the soil can be increased. (Dobeneiner, 1974). Among the three major nutrients required by the plants nitrogen is the most critical nutrient. Nitrogen is the key - element in the synthesis of proteins and other biologically important compounds required for the growth of the plants. Despite the greater abundance of nitrogen in the atmosphere (78.09 percent) it is in the unavailable elemental gaseous form. Of all the living organism, only a few micro organism posses the bio chemical mechanism for converting the inert elemental nitrogen gas into nitrogenous compounds like ammonia and nitrate. Thus the microbes meet their own biological nitrogen need and release the same into the soil enviorонmentfor the utilisation by plant systems. With the spiralling up of the prices of the fertilizers (Poster, 1983) an increasing thrust over the use of Biofertilizers in agriculture has come into practice.

Biofertilizers is the name given to those microbes which can fix atmospheric nitrogen i.e. they are capable of converting atmospheric elemental nitrogen into ammonia. These nitrogen fixers have been estimated on a global scale and the amount of nitrogen fixed by these biological agencies have been worked out to be 175 million tonnes per year in the soil (Tilak, et al 1985). These micro organism also play an important role in the maintanewceof soil fertility by supplementing the nutrients. They have thus created a challenge for all microbiologists and agricultural scientists to explore for new horizons, so that a part of the chemical fertilizer may be replaced by these Biofertilizers. Artificial inoculation of these group of micro-organisms in aspecific area may temporarily or permanently change the balance of the rhizosphere physically, biologically or nutritionally depending on the nature and duration of effects. The classifical example is the Rhizobium legume symbiosis, which has already become a welcome technology in the hands of agriculturists. Apart from this, a number of other species of micro organism effectively fix the atmospheric nitrogen.

Nitrogen fixing biofertilizers are either in the free living condition or in symbiotic association. An important symbiotic association in the rice ecosystem, is that between the blue green algae Anabena azollae and water fern Azolla

pinnata living in the cavities of dorsal lobes of Azolla. This association has now become an important bio fertilizer in rice ecosystem (Kannayian, 1984).

A limited number of heterotropic bacteria play an important role in fixing atmospheric nitrogen as balancing the nitrogen cycle in nature. Free living aerobic bacteria like Azatobacter Beijerinckia, Klebsiella and other members of the anaerobic bacteria clostridium are capable of utilizing atmospheric nitrogen. Blue-ggeen algae such as Nostoc, Aulosina etc., are also capable of fixing nitrogen in paddy soils.

A spiral shaped, gram negative motile vibroid-organism with micro aerophilic nature called Azospirillum is known to have close promixity with higher plants systems. It is remarkable that this organism fixes appreciable quantity of nitrogen (nearly 40kg N/ha/year) in grasses as found by Smith et al (1978). Studies have revealed (Okon et al, 1983, Subba Rao, et al 1982 Wani, 1985), that Azospirillum was of ubiquitous occurence and was claimed to be the most efficient diazotroph among the free living systems. Genus Azospirillum has now been classified into four species according to Terrand et al (1978). The species accomadated are A.lipoferum, A.brasilense, A.amazonense and A.seropideceae.

The bacterium Azospirillum is increasingly becoming popular in many of the developing countries in the world. Inoculation experiments conducted by Dhanapal et al (1978) revealed that root associations of Azospirillum was unique in crops like maize, sorghum, pearlmillet, sugarcane, wheat, in weed plants like guinea grass, digitaria decumbens, amaranthus, cumbu (Smith et al 1976) in xivophytic plants like opuntia (Venkateswarulu, 1982), in plantations like cocoa, rubber, coconut, (Subba Rao et al 1983). Recently rhizosphere of rice, sweet potato, black gram, sunflower, cumbu, barley (Subbarao et al 1979), have known to harbour this organism in the root system. Thus, this bacterium has gained a greater importance in the agricultural field than any other free living bacterium.

Azospirillum was ubiquitous, in the sense it not only increased the grain yield and plant biomass, it was also found in a wide variety of terrestial habitats (Knowles, 1981). Nearly 50 percent of the tropical soils harbour this bacterium, to a remarkably good percentage as compared to the total percentage of heterotrophs in the soil (Döbereiner, 1976). Azospirillum grew well in all types of soil with wide variation in physical properties like pH, temperature and moisture content of the soil. Inspite of the problems of the soil like salinity, alkalinity and acidity influencing the growth of the microbes, Azospirillum

had been proved to be very effective in the alkaline soils (pH 7.5 to 8.5) and acid soils (5.0 - 6.0) (Purushothaman et al 1985).

Azospirillum strains inoculated to different crops showed enhanced acetylene reduction activity (ARA) at low nitrogen content (Nayak et al, 1981). ARA being the index of nitrogenase* activity reveals the high nitrogen fixing capacity of Azospirillum (*Nitrogenase is the enzyme complex that converts atmospheric nitrogen into ammonia in nitrogen cycle)

Strains (Avivi et al, 198X).

Azospirillum strains had the property of excreting the fixed ammonia into their environment. This ammonia was then utilized by the plants. The denitrification propeity of this bacterium reduced the nitrogen availability to plants. (Melado et al, 1982) Denitrification was nothing but the processes of oxidation of the fixed ammonia to nitrite, nitrate and nitric oxide. This was possible with the help of the two enzymes the nitrite reductase and nitrate reductase. These two enzymes thus released the biologically fixed nitrogen into the atmosphere from the soil. This inhibited the nitrogen fixing capacity of the bacteria.

Azopirillum isolates were also found to produce growth promoting substances like indole acetic acid (IAA)

gibberlins, cytokinins (Phillips, 1970). These growth promoting substances were found to increase the plant growth (Silveria et al 1983). Azospirillum spp produced indole acetic acid even in the absence of tryptophan in the medium (Moore, 1980), where tryptophan was the precussor of indole acetic acid.

Azospirillum, association in various plant roots from rice to cocoa (Subba Rao, 1982) and in various physiological enviornments had made possible, the application of Azospirillum inoculant to various plants and various soils.

India being a developing country with various types of soils (Porter, 1963) requires these types of bio-fertilizers in large quantities. Soil physiological properties of the soil influence the growth of the micro organisms. Acidity of the soil may prevent the growth of micro organisms. Azospirillum had been proved to grow well in both acidic soils (Smith, 1984) and alkaline soils (Purushothaman, 1984). In Tamil Nadu Azospirillum inoculation had been proved to be very effective in most of the soils (Narayanan, 1981).

In this study Azospirillum was isolated and characterised from acid soils of Kuttanadu and Trichur of Kerala State. A growth of Azospirillum at acidic pH in the range of 4.0 to 5.0 has been conducted in order to inoculate Azopirillum

to highly acidic soils. Further experiments were carried out to study the effect of nitrogenase activity and denitrification capacity of the isolated species. Studies were also conducted on the ability of the Azospirillum isolates to produce phytohormones and excrete ammonia. Azospirillum species were also inoculated to peat soils of pH 5.0 - 5.5 and its effect on plant growth was studied.

Thus in the present study the possible use of the bio-fertilizer Azospirillum to acidic soils have been evaluated.

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REVIEW OF LITERATURE

Biofertilizers are used in modern agriculture primarily to supplement the need for chemical fertilizers. There is fast depletion of the natural gas, coal and minerals and oil resources which form the basis for the production of chemical fertilizers. This resulted in the escalation of the cost of these chemical fertilizers. With all these difficulties, it has become a challenge for all the agricultural microbioligists to explore new horizons, so that a part of these chemical fertilizers are replaced by biofertilizers. The microbes play an important role in the maintenance of the soil fertility. It has been estimated that the amount of Nitrogen fixed by biological agencies works out to 175 million tonnes per year per hectare and it was found that most of this nitrogen fixed was due to the legume Rhizobium system (Burns and Hardly, 1973) Careful studies on the symbiosis of Rhizobium with legumes revealed that not only more nitrogen was fixed, but also the yield of pulses increased. (Elango, 1981, Rovira 1965, Stern et al, 1980)

Apart from Rhizobium system, it has been proved beyond doubt that biological Nitrogen fixation takes place in fields, forests and other green lands (Evans and Barber, 1977). Free living aerobic bacteria like Azatobacter,

Beijerinckia and the anaerobic bacteria Clostridiewm spp. Contribute to these processes. Algal association like Azolla-Anabena, nodulated non-legumes like Alnus Spp. were also reported to be potential sources of nitrogen fixation (Evans and Barber 1977). Thus increasing the dependence of biologically fixed nitrogen is an alternative to reduce the utilization of costly nitrogen fertilizer. Seed inoculation with appropriate strain has been an accepted technology nowadays. (Stump et al., 1977).

This discovery of nitrogen fixing organisms on roots of grasses including maize, wheat, pearl millet, sorghum has led to speculations about the possibility of providing nitrogen for important crops by the use of associative systems. Here plants cannot fix nitrogen directly but provide energy to the associate bacteria which in turn provides nitrogen to the plant in the ammoniacal form. This association was found in many tropical plants with high nitrogenase activity and are characterised by the C_4 Photosynthetic pathway with minimal loss of carbon to photo respiration. These bacteria were given the name Azospirillum. Considerable progress have been made towards understanding the Azospirillum species and the review auguments its significance as a potential diazotroph in grass eco system (Okon, 1984).

ASSOCIATIVE SYMBIOSIS AND NITROGEN FIXATION: In some plant geno-types which are efficient photosynthesizers, the root

exudates are utilised by certain bacteria. This is, in other words referred to as associate symbiosis. It was later termed as BIOCOENOSIS in the international workshop of nitrogen fixation held at Brazil (Dobereiner and Depolli, 1980) certain forage grasses, rice and sugarcane stimulated the multiplication of BEIJERIENCKIA and decreased the number of amino acid requiring bacteria, molds actinomycetes in the rhizosphere and thus the sugarcane Beijerienckia biocoenosis are well documented (DOBEREINER and DEPOLLI, 1980). Similar rhizocoenosis of wheat with the BACILLUS species (Larson and neat, 1978) and ACHROMOBACTER like organism with rice (Watanabe and Barraquio 1979), Paspalum notatum association with Azotobacter Paspali was reported.

The Azospirillum rhizocoenosis was first reported in digitaria grass (DOBEREINER and DAY, 1976) and number of papers have been reported on the rhizocoenosis of SPIRILLUM LIPOFERUM with many species (Laksmikumari et al 1976). Diazotrophic spirullum like organism were isolated from roots of number of C_4 plants mainly grasses. Later Azospirillum association in maize, rice and wheat was proved with host plants specificity (BALDANI and DOBEREINER, 1980). RECOGNITION OF AZOSPIRILLUM: Azospirillum was first described by Beijerinch (1930) as Azotobacter spirillum, which apparently fixed nitrogen in enrichment cultures and was later named Spirillum lipoferum which was isolated from

a number of soil samples in Australia and Germany (SCHRODER 1932). The same organism was identified by number of scientists and these cultures incorporated $15N_2$, in pure culture when grown in yeast extract. (Ronnie et al, 1980).

In 1974, Dobereiner proved the symbiotic association between Digitaria decumbens and S.lipoferum and it was reported that S.Lipoferum was a very common root inhabitant in tropics (Dobereiner et al, 1976) and organism was assigned a new genus Azospirillum (Tarrand et al, 1978) comprising two species A. liproferum and A. barsilense which is now a genus of four species (MAGALHAES et al, 1983). 48 percent of the cultures in this genus was found to be tropical plants and were mostly the above two species (Rence.M kosslak et al, 1983).

BIOGEOGRAPHY OF AZOSPIRILLUM: The organism was found very common in tropical, temperate, and sub-tropical soils (DAS et al, 1980) Azospirillum has been isolated in maize (Dhanapal et al, 1978). Wheat (Nery et al, 1978) Pearl millet, Sorghum (Dhanapal, 1978) Sugarcane (Hegazi et al, 1979) Cotton (Purushothaman, et al, 1980) and in number of grasses (Mellado et al, 1980).

Reyenders and Vlassak (1978) found no relation between soil characteristics such as pH, texture and the occurence of Spirillum in different plant species.

Occurence of Spirillum species, in various geographical regions was reported by Amar et al (1980). In India Rao et al (1978) reported the ubiquitous presence of the spirillum in different soil types. Azospirillum occurence was found to be poor in European soils (DESALVIO et al (1978). Reports indicate the occurence of spirillum in China and also in Polish soils (Kulinska et al, 1983). In Tamilnadu Azospirillum was found in most of the soils except the soils of Nilgiris, alkaline soils of Trichy (Anonynous, 1981). Azospirillum was found in most of the soils and was isolated from sweet potato (Hill et al, 1981), Amaranthus (Udayasuriyan, 1981), Opuntia (Rao, et al, 1982) and Barley (Moorthy, 1981).

FACTORS INFLUENCING THE OCCURENCE OF AZOSPIRILLUM:

A number of factors influence the occurence of Azospirillum in soils. PH around 7.0 was found to be optimal. Sporadic occurence was noted in soils of PH 4.8. (Doberginer et al, 1976). Activity of Azospirillum was found to increase with increase in temperature. Nitrogen fixed by the same species at 20 degree centrigrade was only half of that at 30°C (Scott et al, 1978). Azospirillum was an aerobic organism fixing nitrogen at micro-aerophilic condition. (Scott, 1978). In some plants, the organism was associated with the organic matter content. Moisture level and organic matter limits the growth of the bacterium in soil. Various carbon sources also influence the growth of the Azospirillum (Amaresh Das et al, 1983). Soil management practice also influence Azospirillum species (Charyulu, 1980), Barber et al

(1979) reported that spirillum species was not influenced by the fixed nitrogen. Most of the Azospirillum species in different soils were found to use EMP* for their Catabolic pathways (Westby et al, (1983). Suboptimal levels of nitrogen fertilizers increase the nitrogen fixing ability of the Azospirillum (Kapulnik et al, 1981).

TAXONOMIC CONSIDERATIONS:

Breed et al, (1957) classified Spirillum lipoferum under the genus spirillum in the order Pseudomonadiles. This is a gram negative motile bacterium, Vibroid in shape containing poly β hydroxdy butyrate granules (Dobereiner and Daym 1978) Salts like malate, succinate lactate pyruvate, Fructose have been found to be oxidisable carbon source (Okon et al, 1976). A number of scientists thought that there was 3 species of Spirillum lipoferum at first (Sampdio, 1978) and so was classified with two species in the genus Azospirillum (Kneig and Tarrand 1978) based on DNA homology and physiology tests. They are A.lipoferum and A.Brasilense with reference to their r-RNA cistrons which differ from spirilla (Smedht, et al, 1980). Now after four years during the study of the occurence of this genus in Amazon soils, the third species in the Azospirillum was identified with the similar characterists, but more sensitive to alkaline medium (Magalhaes et al, 1984) and was called A.amazonense. The fourth species was identified during the survey of Azospirillum

EMP* - Glycolysis.

association with various cereal roots, as dry white colonies and was distinguished from other species and named A. Seropedicae (Baldani et al, 1984).

Dobereiner et al (1980) reported that both the former species contain nir^{+}* and nir^{-}* groups i.e. those which show high denitrification while in the other there is no apparent disappearance of nitrogen. Serological analysis showed the difference in the subgroups and the species in the genus Azospirillum (Depolli et al, 1980).

EFFECT OF AZOSPIRILLUM INOCULATION ON NITROGENE FIXATION:

A correlation between acetylene reduction activity of the roots of maize and enrichment cultures of Azospirillum concluded that Azospirillum species were largely responsible for nitrogen fixation in grasses because of their occurance in the root tissues (Day et al, 1976) which has now been confirmed by a number of microbiologists (Lakshmikumari et al, 1976), Okon etal, 1976).

S.lipoferum inoculated grasses gave significant yield response over uninoculated control (Smith et al, 1975). S. lipoferum inoculated corn, sorghum, millet and other grasses showed increased nitrogense activity dry weight and total nitrogen over uninoculated control (Okon et al, 1976) In China S. lipoferum inoculation, showed increased Nitrogenase activity in digit grass (Ling & Won, 1978). Azospirillum inoculation of Sorghum in Israel resulted in increased grain yield (Kapul-nik, 1984).

* nir^{+} - nitrification property.

* nir^{-} - denitrification property.

Combined inoculation of Azospirillum and Azatobacter to maize and sorghum showed beneficial effects in pot culture experiments (Subba Rao et al, 1982).

$^{15}N_2$ incorporation and acetylene reduction activity studied on maize strains (Rennie, 1980) and rice (Charyulu, 1981) confirmed the nitrogen fixation with Azospirillum. When nitrogen fixation of corn after inoculation with Azospirillum was studied acetylene activity increased before maturity and then decreased till harvest (Alberecht, 1981). Inoculation of pearl millet resulted in significant growth and dry matter, under sterilized and unsterilized condition (Venkateswaralu et al, 1983). Pearl millet with 75% nitrogen fertiliser and Azospirillum showed high yield over 100% fertilizer nitrogen and Azospirillum inoculation (Purushothaman et al, 1979).

Dhanapal et al (1978) reported that seed inoculation increased vigour index and seedling growth of Combu, Maize, Sunflower, Cotton and Rice. Seed inoculation of A.brasilense with 45kg nitrogen per hectare to Sorghum gave similar results. (Singh et al, 1980, Ramak Pal et al, 1981). Inoculation experiments conducted in Florida with A.brasilense to slightly acidic soils showed and increased acetylene reduction (Smith et al, 1984). Response of A. brasilense to wheat under field condition showed increase in nitrogen fixation, fresh root and shoot weight and yield (Kundu et al, 1983).

Effect of Azopirillum inoculation to other millets like ragi was also encouraging (Muthukrishnan, 1981). A. brasilense inoculation to grasses like setaria italica showed increased acetylene reduction and nitrogen fixation (Okon et al, 1983). Azospirillum inoculation to Cumbu showed 40-60% increase in grain yield (Anonymous, 1981). Increase in yield in rice with Azospirillum inoculation was reported by Natarajan et al, (1980). Use of Azospirillum as a biofertilizer for cotton saved nitrogen fertilizer (Purushothaman et al, 1981).

PLANT ROOT INFECTION AND ESTABLISHMENT:

Azospirillum species had a close association with roots than its independent activity in soil (Dobereiner, 1975) and emited growth substances (IAA, Cytokinim) (Tien et al, 1979) caused root hair multiplication and shortening of the roots and also thicken the roots (Umali et al, 1978) and enhanced mucigel productions and the organism was embedded in it in the presence of fertilizer nitrogen. In the absence of mineral nitrogen, Azospirillum adheres to root base. In seedlings Azospirillum invades the root via middlelamelle of older root tissues and transparent area of the invading cells reveal the hudrolysis of plant all wall (Umali Garcia, 1978).

In maize there was an increase in the number of Azospirillum from 10^4 to 10^7 per gram root (Magalhaes et al, 1978) and the infection was found to spread to stem in maize

and wheat Watanabe and Barraquio, 1979). A number of scientists (Henson et al, 1977, Okon et al, 1980) reported on the production of pectiolytic enzymes by Azospirillum strains and their utilization of pectin as carbon source. Rhizosphere and inner rhizosphere of grasses were found to harbour a dence population of Azospirillum (Purushothoman 1980). Thus Azospirillum species were determined in rhizosphere and roots of most of the creals (Mellado, et al, 1983).

PLANT GROWTH PROMOTING SUBSTANCES:

Strong evidences on the production of indole acetic acid (Brown, 1972) gibberlins (Burlingham, 1968) and cytokinins (Phillips, 1970) by bacteria, were essential to the growth and development of plants. These growth hormones were also found to increase the growth rate of plants (Barea et al, 1976).

Azospirillum was also found to produce Auxin (Burris, 1976) which increased or suppressed the growth of plants (Gaskins et al 1977). Cytokinins and other plant growth harmones produced by this organism was found to increase the dry weight of the plant (Hubbel et al, 1979).

IAA and similar compounds were produced by A.brasilense from tryptophan in culture condition. (Stearn, 1980). Along with this a small quantity of Gibberlins and cytokinins were also detected. Azospirillum produced IAA with or without tryptophan addition to the medium. High concentration of IAA was also found to be inhibitory to nitrogenase activity in many cases (Silveria et al, 1983).

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Experimental Procedures

EXPERIMENTAL PROCEDURES

The nitrogen fixing bacterium, Azospirillum was isolated from the acid soils of Kerala State. Azospirillum, was found to be a very effective nitrogen fixing bacterium and the various experiments conducted with the three soil samples collected from Kuttanad and Trichur of Kerala State are discussed below. The soils were collected before five months. Experiments were also conducted with the Azospirillum spp isolated from these soils and the procedures are discussed below.

I. ISOLATION OF AZOSPIRILLUM (DAY AND DOBREINER, 1976).

1) SOURCE OF THE SOIL: Typical acid soil samples from Kuttanad, and Trichur of Kerala State were collected and brought to the laboratory. In 1:1.5 (W/V) soil water solution, these soil samples recorded a pH of 5.0 to 5.2. The soil samples were taken in 10cm circular plastic pots and surface sterilized seeds of Sorghum (Sorghum bicolor) Va. Co 24 were sown. The seedlings were allowed to grow for thirty days.

ii) ISOLATION OF AZOSPIRILLUM: The seedlings were uprooted and the roots were collected, incised, washed in running tap water and cut into small bits. The root bits were surface sterilized with 80 percent ethyl alcohol and washed with changes of sterilized distilled water. The root bits

were plunged into test tubes containing aspectically semi-solid malate medium (IV a) supplemented with 100 mg of glutamic acid or 50mg of yeast extract per litre. After 48 hours of incubation at room temperature (28ºC), Characteristic white pellicles was transfered to another test tube containing nitrogen-free malate medium (NFb) and observed for the development of subsurface pellicles. For purification, loopful of the pellicles was streaked in NFb agar plates with glutamic acid as nitrogen source. After 5 days, typical small white dense colonies were picked and transferred to semi-solid NFb medium. Final purification of the isolates was done by streaking on potato (IV d) agar (BMS) medium and the typical pink wrinkled, raised colonies were transferred to agar slants.

iii) INDENTIFICATION: Identification of the isolates of Azospirillum was made, following the scheme proposed by (Tarrand et al, 1978) in addition to the following diagnostic aids:

a) Small white pellicle formation at subsurface.
b) Change in colour of the medium from straw yellow to brilliant blue.
c) Positivity of Nitrogenase activity (ARA).
d) Microscopic examination of 72 hours old culture revealing the typical spiral movement.

iv) COMPOSITION OF THE MEDIUM USED:

a) NITROGEN FREE MALATE MEDIUM (NFb) (DOBEREINER, 1980).

Malic acid	..	5.0g
Diptossium hydrogen phosphate	..	0.5 g
Magnesium sulfate	..	0.2 g
Sodium chloride	..	0.02 g
Sodium molybdate	..	0.002 g
Manganese sulfate	..	0.01 g
Fe EDTA (1.64 per cent W/V, Aqueous)	..	4.0 ml
Bromothymol Blue (0.5 percent W/V inethonol)	..	3.0 ml
Potassium hydroxide	..	4.5 g
Trace element solution	..	2.0 ml
Vitamin solution	..	1.0 ml
Distilled water	..	1000 ml.
pH	..	6.8 (adjusted with in Sodium Hydroxide.
Agar	..	15 g 1.75 g for semi-solid medium.

COMPOSITION OF TRACE ELEMENT SOLUTION:

Magnesium sulfate	..	150 g
Copper sulfate	..	15.0 g
Zinc sulfate	..	8.9 g
Boric acid	..	0.5 g
Sodium molybdate	..	0.5 g
Ferrous sulfate	..	20.0 g
Citric acid	..	20.0 g
Distilled water	..	1000 ml.

COMPOSITION OF VITAMIN SOLUTION:

Biotin	..	10.0 mg
Pyridoxine	..	20.0 mg
Dist. water	..	100 ml.

COMPOSITION OF POTATO MALATE AGAR MEDIUM (BMS)

Potatoes	..	200.0 g
Malic acid	..	2.5 g
Potassium hydroxide	..	2.0 g
Sucrose	..	2.5 g
Vitamin solution	..	1.0 ml
pH	..	7.0

Washed potatoes (200g) were cooked for 30 minutes and the solution was then filtered through muslin cloth. Potassium malate was prepared by dissolving 2.5 g of malic acid in 50ml. of dist. water and adding 2.0 g of potassium hydroxide adjusting the pH to 7.0. To the potato filterate, potassium malate, sucrose and vitamin solution were added and diluted to 1000 ml with dist. water.

II. POPULATION STUDIES OF MICRO-ORGANISMS IN THE SOIL: (PRAMER AND SCHMIDT, 1966)

The acidic soil under study, collected from the Kerala State with pH 5.2, was found to contain 0.8 percent of organic content. Weighed quantity of the soil sample was placed in Erlenmeyer flasks containing 100ml of sterile distilled water and placed over a gyrotary shaker for bringing the micro organism into suspension before the serial dilution was made.

i) ESTIMATION OF TOTAL HETEROTROPIC BACTERIA:

From the serial dilution prepared from soil sample one ml of 10^{-7} dilution was pipetted out into a sterle petriplate and yeast extract glucose agar medium of the following composition was poured after sterilization:

Peptone	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Glucose	5.0 g
Trace element solution	1.0 ml
Agar	15.0 g
Distilled water	1000 ml
pH	7.0

The plates were incubated for 72-96 hour at 30°C and the number of bacterial colonies were counted and expressed as number of colonies per gram of soil on dry weight basis.

h) ESTIMATION OF DIAZAOTROPH, AZATOBACTER:

From the serial dilution made from soil sample, one ml of of either 10^{-2} or 10^{-3} dilution was pipetted out in a sterile petri-plate and Waksman medium 77 of the following composition was poured after sterilization:

Mannitol	10.0 g
Sodium chloride	0.2 g
Magnesium sulfate	0.2 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulfate	0.2 g
Maganese sulfate	Trace
Ferric sulfate	Trace
Zinc sulfate	0.0005g
Copper sulfate	0.0002 g
Sodium molybdate	0.001 g
Agar	10.0 g
Dist. Water	1000 ml.
pH	7 ± 0.1

The plates were incubated and the number of colonies formed per plate was counted and expressed as number of colonies per gram dry weight of soil.

C) ENUMERATION OF AZOSPIRILLUM:

For enumeration of Azospirillum in the soil sample, most probable number technique (MPN) was used (Okon et al, 1977). Semi-solid malate medium was employed in test tubes in replication. From the serial dilutionmade from soil sample 1.0 ml of 10^{-7}, 10^{-6} and 10^{-5} dilutions were transferred to the tubes containing the medium. The estimates were derived from statistical tables of Cochram (1950) and expressed per gram dry weight of soil.

III. GROWTH RATES OF DIFFERENT ISOLATES OF AZOSPIRILLUM:

An invitro experiment was set up to trace the growth rate of different Azospirillum isolates. A quantity of 100ml of yeast extract glucose broth was dispensed and sterilized and inoculated with standardised cell suspension of Azospirillum. The flasks were placed over shaker for 24hours at 30°C. Aliquots were drawn from the flasks at 3 hours intervals and their optical density values, determined at 490 mm in a spectro photometer (Schimedizer, Japan). The growth rate and generation time of the isolates were determined by plotting the values on a semi-log graph. The growth yield of the isolate were determined by transferring 2.0 ml of the broth into weighed aluminium planchets and dried at 80°C for 8 hours.

YEAST EXTRACT MEDIUM:

Glucose	...	5.0 g
Peptone	...	5.0 g
Sodium chloride	...	5.0 g
Yeast Extract	...	3.0 g
Dist. Water	...	1000 ml
pH	...	7.0

6) EFFECT OF PH ON GROWTH OF AZOSPIRILLUM ISOLATES:

An in-vitro experiment has been set up to trace the growth of two isolates of Azospirillum at pH 4.0 .Yeast extract glucose broth, adjusted to pH 4.0 after sterilization in 250ml Erlenmeyer flasks were inoculated with the inoculum. The flasks were placed over a shaker (New Baraskrick, USA) at 30°C. A liquots were drawn at 4 hour intervals and the optical density was determined at 490nm anddetermined by plotting over a semilog graph.

IV. PHYTOHORMONE PRODUCTION BY AZOSPIRILLUM ISOLATES:

One set of malate broth (NF b) in 70 ml quantities was sterilized in Erlenmeyer flasks and then added 0.2 ml of the inoculum and incubated at 30°C over a gyrotary shaker (150-160 strokes/min) suitable control was also maintained. After 5 days of growth the cells were spun down by the centrifugation at 5000G for 15 minutes.

a) EXTRACTION AND ESTIMATION OF INDOLE ACETIC ACID (IAA)
(Chandramohan and Mahadevan, 1968, Tein et al, 1979)

The cell free culture filterate was adjusted to pH 2.8 with 1N hydrochloric acid and taken in a separating funnel. Equal volume of peroxide free cold ether (4°C) were shaken and allowed to stand at 4° C with intermittent shaking. Aqueous phase was seperated from organic phase. At 4 hour intervals, 2 more extractions were done and organic layers were pooled together and evaporated to near dryness. The residue was dissolved in 2.0 ml of distilled methanol.

b) DETERMINATION OF TOTAL INDOLE ACETIC ACID:

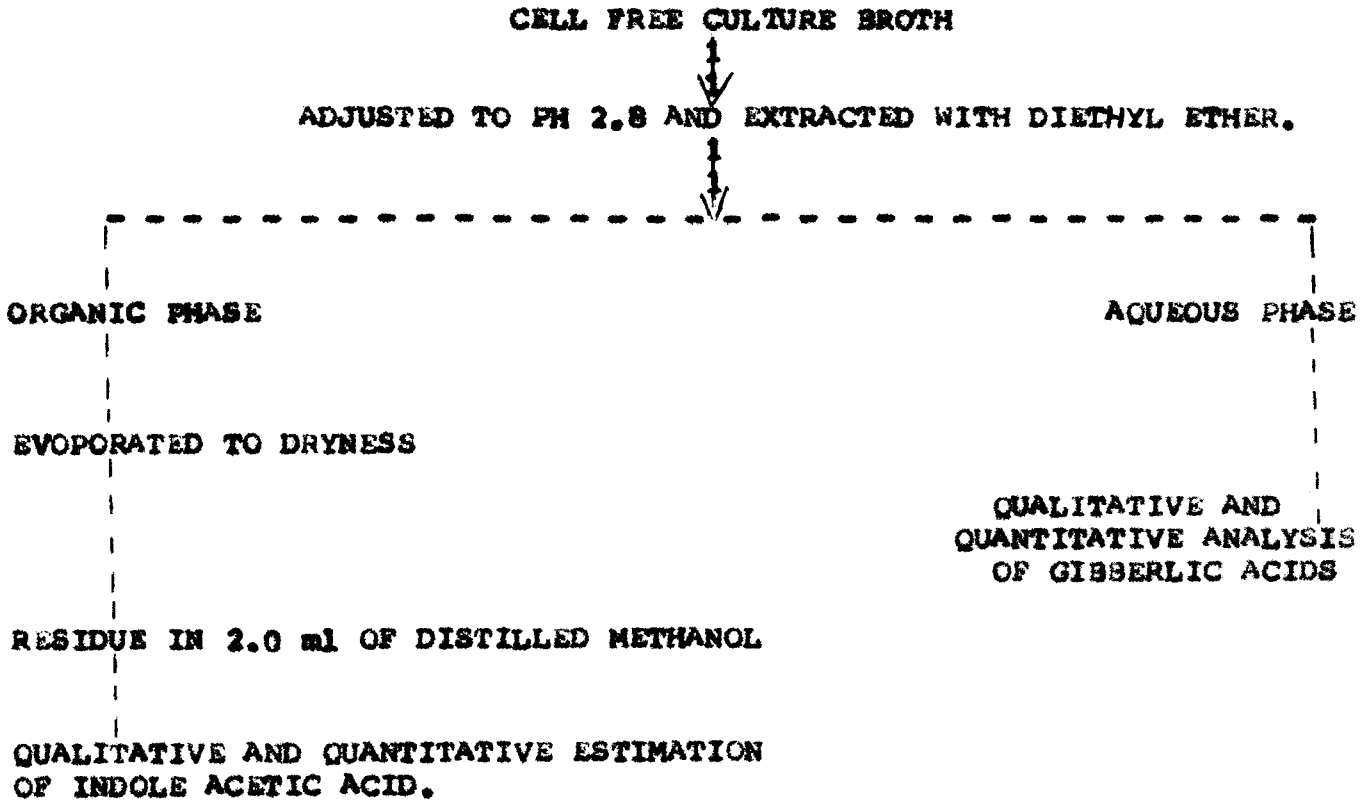
The quantity of IAA was estimated using the Salper's reagent containing 1.0 ml of 0.5 M ferric chloride in 50.0ml of 35% per chloride. To 0.5 ml of the sample in the test tube, 1.5 ml of distilled water followed by 4.0 ml of salperls reagent was added and incubated in darkness for 1 hour at 26°C. The intensity of the colour developed was measured at 535nm in a spectrophotometer and the quantity of IAA in the sample was determined.

c) CHROMATOGRAPHIC IDENTIFICATION OF INDOLE ACETIC ACID:

A quantity of 100 Micro litre of the samples were spotted on whatman No.1 Chromatographic paper. (27 x 35cm). The chromotogram was developed ascendingly in a solvent of isopropyl alcohol: ammonia : water 10:1:1 (V/V). Then the chromatogram was air dried and observed under UV light for

the occurence of Fluorescent spots using an all glass atomizer, the paper was sprayed with Salkowshi's reagent (Dullaart, 1967) containing 1.0 ml of 0.5 M. ferric chloride in 50 ml of 5% per chloric acid. Then the paper was air dried and heated at 60°C for 3 to 5 minutes. The colour of the spot and the RF values were compared with that of the standard that was co-chromatographed.

FLOW DIAGRAM FOR EXTRACTION OF INDOLE ACETIC ACID;



NITROGEN FIXING CAPACITY OF THE ISOLATES:

(a) ACETYLENE REDUCTION ACTIVITY OF THE ISOLATES (ARA) Bugerseak, 1981.

Nitrogen free semi-solid malate medium (Baldani and Dobereiner, 1980) was prepared in 30 ml quantities in serum bottles. The vials were inoculated and incubated at 30°C in static condition. After 5 days of growth, cotton plugs were removed and replaced by tight fitting needle puncture rubber stoppers. The air in the flask was removed by syringing out and 1 percent acetylene gas was injected. The flasks were incubated at 30°C in static condition without disturbing the pellicles for 6 to 12 hours. A quantity of 0.5 ml of the gas sample was withdrawn through a sterile disposable syringe and fed into a gas chromatogram (Perkin and Elemer, USA) having FID system with poropack column (80 to 100 mesh). The ignition temperature was 160°C and oven temperature was 95°C. The flow rate of the carrier gas nitrogen was 20 lb/min, hydrogen 16 lb/min and oxygen 25 lb/min. The peak length of the ethylene was noted. The nitrogenase activity was obtained applying the formula.

$$\text{Nitrogenase activity} = \frac{\text{Chart units} \times \text{attenuation} \times \text{Vol. of gas phase flask}}{\text{Hrs. of incubation} \times \text{Vol. of gas sample injected into the chromotogram.}}$$

The nitrogenase activity was expressed as n moles of ethylene produced per hour per mg of protein. (Hardy et al, 1951).

6) PROTEIN ESTIMATION IN THE CELL: (LOWRY et al, 1951).

The cells in the broth was first digested. One ml of the homogenised culture broth was pipetted out from the vial into a boiling tube and 2 ml of 10 percent potassium hydroxide was added and boiled over a water bath for 5 minutes, till the solution becomes clear. The clear solution was used for the estimation of protein.

c) COMPOSITION OF REAGENTS:

a) Alkaline Copper solution: 2%

A quantity of 50.0 ml of the reagent A was mixed with 1.0ml of reagent B at the time of use.

Reagent A: 2 percent sodium carbonate in 0.1 N sodium hydroxide solution.

Reagent B: Copper sulfate 0.5 percent in 1 percent sodium tartarate.

b) FOLIN-CIOCALTEAU REAGENT (1 N).

1.0 ml of the sample was taken in a test tube and made upto 4.5 ml with distilled water. Then 5.0 ml of the alkaline copper solution was added to each tube and mixed well. Then 0.5 ml of Folin-ciocalteau reagent (1N) was added, mixed well and allowed to stand for 30 minutes. The blue colour formed was measured at 620 mm in a spectrophotometer against a blank. The protein content was estimated by referring to a standard graph prepared with bovine serum albumin.

VI. a) NITRATE REDUCTASE ACTIVITY
(YORDY AND RUOFF, 1981).

The Azospirillum isolates were grown in liquid malate medium supplemented with 0.5 percent sodium nitrate and 1.0 percent of lactose. This was incubated for 5 days in shake culture conditions. The broth was centrifuged and the supernatant was collected. To 0.5 ml of supernatant, 1.0 ml of 1 percent sulphanilamide in 1.5 N hydrochloric acid and 1.0 ml of 0.002 percent N-(1-naphthyl) ethylene diamide hydrochloride were added. The intensity of the pink colour developed was read at 540 nm in a spectrophotometer.

b) NITRATE REDUCTASE ACTIVITY: (Yordy and Ruoff, 1981).

The Azospirillum isolates were grown in liquid malate medium supplemented with 10 mg of sodium nitrate. This was incubated for five days under shake culture conditions. The broth was centrifuged and the supernatant was collected. To 0.5 ml of supernatant, added 1.0 ml of 1 percent sulfanilamide in 1.5 N hydrochloric acid and 1.0 ml of 0.002 percent N-(1-naphthyl) ethylene diamide hydrochloride were added. The total volume was made upto 20.0 ml with distilled water. The intensity of the pink colour developed was read at 540nm in a spectrophotometer. The nitrite reductase activity was calculated using the factor, 1 percent light absorbed in equivalent to 5.28 Micro Gram of nitrite.

VII. AMMONIA EXCRETION BY AZOSPIRILLUM ISOLATES: (Bergersen, 1961):

The Azospirillum isolates were grown in 100ml quantity of malate free medium (NFb) in shake culture condition. A suitable quantity was acid digested and an aliquot was carefully pipetted out into a Erlenmeyer flask and the pH was adjusted to 4.0 with sodium hydroxide. The flasks were closed with stopper, fitted with glass rod dipped in 10 perent sulfuric acid after adding 1.0 ml of 20 percent sodium hydroxide. The flasks were left overnight avoiding position that may cause condensation with the flask. Next morning, the stoppers were removed and the tip of glass rods were dipped in 1.0ml of dist. water in a test tube and treated with 1.0 ml of Nessler's reagent and 4.0 ml distilled water. The intensity of the colour developed was read at 490 nm in a sms spectrophotometer.

VIII. INFLUENCE OF AZOSPIRILLUM INOCULATION ON PLANT GROWTH:

Seeds of sorghum bicolor (Variety Co. 24) and maize (variety Ganga-5) seeds were surface sterilized with 80 percent ethanol for about 5 minutes and washed with sterilized distilled water successively. The seeds were inoculated with different isolates. The inoculum was done by adding 5.0 ml of broth culture of Azospirillum. The seeds were sown in peat and sandy soil mix. (PH Near 6.00) taken in 10cm diameter plastic pots. After 20 days of growth, the seedlings were pulled out, washed free of the soil. The root length, shoot length, fresh weight and dry weight of the plants were determined. Control plants were grown without Azospirillum inoculation. Each treatment had enough replications, permitting statistical secretanity of the data.

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Results and Discussion

RESULTS AND DISCUSSION

Azospirillum inoculation to acidic soils was studied. These organisms were isolated from the acid soils collected from various lands in Kerala State. These isolates were tested for the various activities and were inoculated in pot cultures of maize and sorghum grown in peat soil and the resultsof the various experiments carried out with the acid soil isolates of *Azospirillum* are discussed under following titles:

I. Quantification of total bacteria and diazotrops in the acid soils.

II. Identification of the isolated species of *Azospirillum*.

III. Growth of *Azospirillum* at pH 5.0 and pH 4.0.

IV. Phytohormone production by *Azospirillum* isolates.

V. Acetylene reduction activity (Nitrogenase) of the isolates.

VI. Nitrate reductase (NR) and nitrite reductase (nir) activity of the isolates.

VII. Effectof *Azospirillum* inoculation to maize and sorghum plants and their ammonia excreting capacities of the isolates.

The resultsof the above experiments are given in the following tables and the table I gives the total heterotropic bacteria present in the soil samples.

QUANTIFICATION OF TOTAL HETEROTROPIC BACTERIA IN ACID SOILS

TABLE I-a.

Soil Samples	Dilution	Colonies per plate.	Total Heterotorphs.*
1	10^{-7}	120	150×10^7
2	10^{-7}	114	142.5×10^7
3	10^{-7}	120	150×10^7
		Mean	148×10^7

* Total heterotrophs present per g of soil in dry weight basis.

The results of the quantitative analysis of the three soil samples for total heterotophic bacteria are presented in table Ia. The data indicates that all the three acid soils recorded fairly high populations of heterotropic bacteria, inspite of the pH of the soil samples ranging between 5.0 to 5.2. The total heterotrops registered on an average 14.8×10^8 per gram dry weight of the soil.

The population of the heterotrophs in various soils normally ranges from 9.0 to 10.0×10^8 counts per gram of soil on an average (Subba Rao, 1979). From the above date it was clear that thepopulation of heterotrophs in all types of the soil samples tested are almost similar.

QUANTIFICATION OF AZATOBACTER IN ACID SOILS

TABLE Ib.

Soil Samples	Dilution	No. of Colonies	No. of Azatobacter*
1	10^{-3}	72	90×10^3
2	10^{-3}	64	80×10^3
3	10^{-3}	78	96.5×10^3
		Mean	89×10^3

* No. of Azatobacter present in 1g of soil sample.

Table Ib represents the population of nitrogen fixing diazothoph Azatobacter in the soil samples. Azotobacter counts according to this data were not very high, but restricted to a range of 1000 to 10000 per gram of soil. The data recorded on an average 8.9×10^4 cells per gram of soil samples on dry weight basis.

The above population of the diazotroph, Azatobacter was very low when compared to the normal population of Azotobacter which ranges from 80 to 90×10^4 counts per gram of Soil. The data has clearly indicated, the inability of the bacterium to survive in acidic pH.

QUANTIFICATION OF AZOSPIRILLUM FROM ACID SOILS

TABLE II

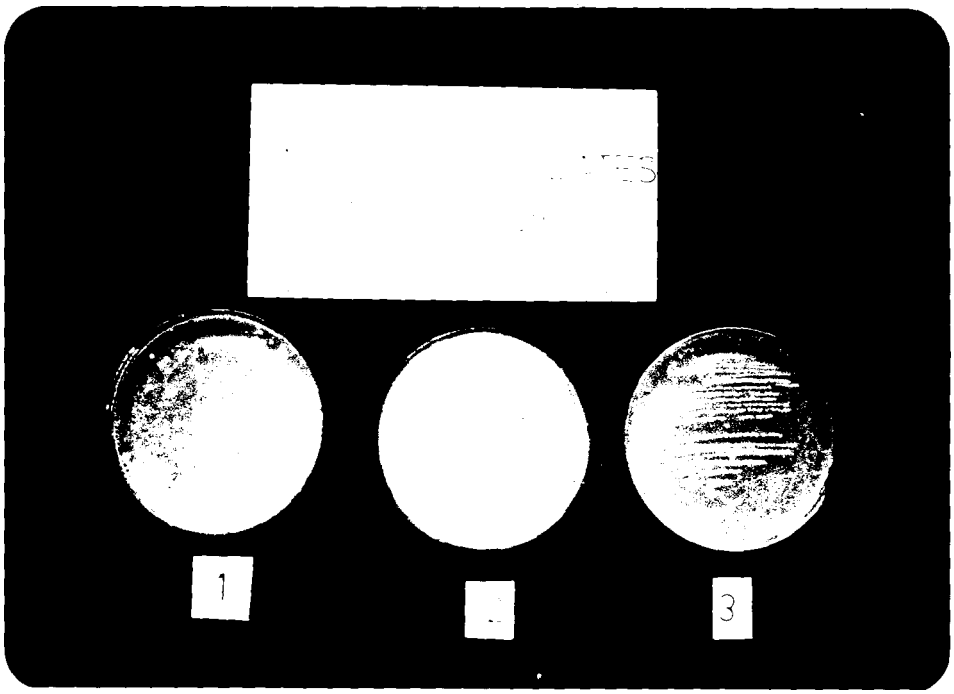
SOIL SAMPLES	POPULATION OF AZOSPIRILLUM*
1	53.75×10^5
2	6.25×10^5
3	7.75×10^5
Mean	22.58×10^5

* No. of counts per gram of soil.

The microbiological analysis of soil samples obtained from Kuttanad, Kerala State for the occurence of Azospirillum is presented in Table II. All the three soils samples harboured Azospirillum in fairly large numbers. On an average the soil had 22.58×10^5 counts of Azospirillum per gram of soil in dry weight of soil. Azospirillum population constituted 0.15% of the total heterotropic flora of the soil.

Inspite of the acidic nature of the soil Azospirillum counts were fairly high, when compared to the normal population of Azospirillum in tropical soil, $30-40 \times 10^5$ cells per gram of soil at neutral pH (Subbaroad, 1979).

PLATE : I



ISOLATES OF AZOSPIRILLUM IN AGAR PLATES

PLATE : II

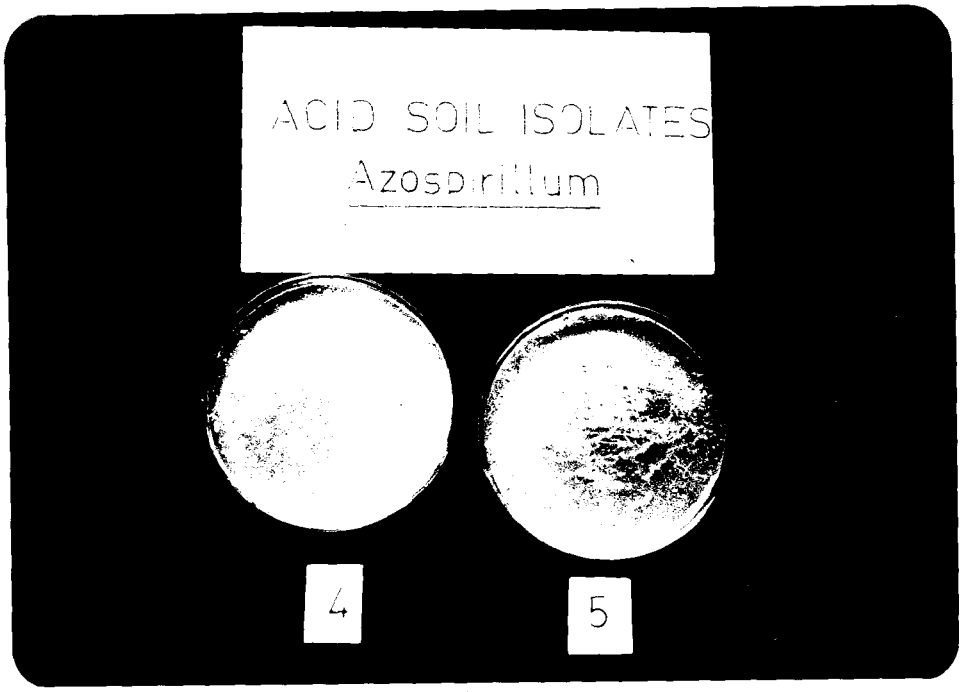
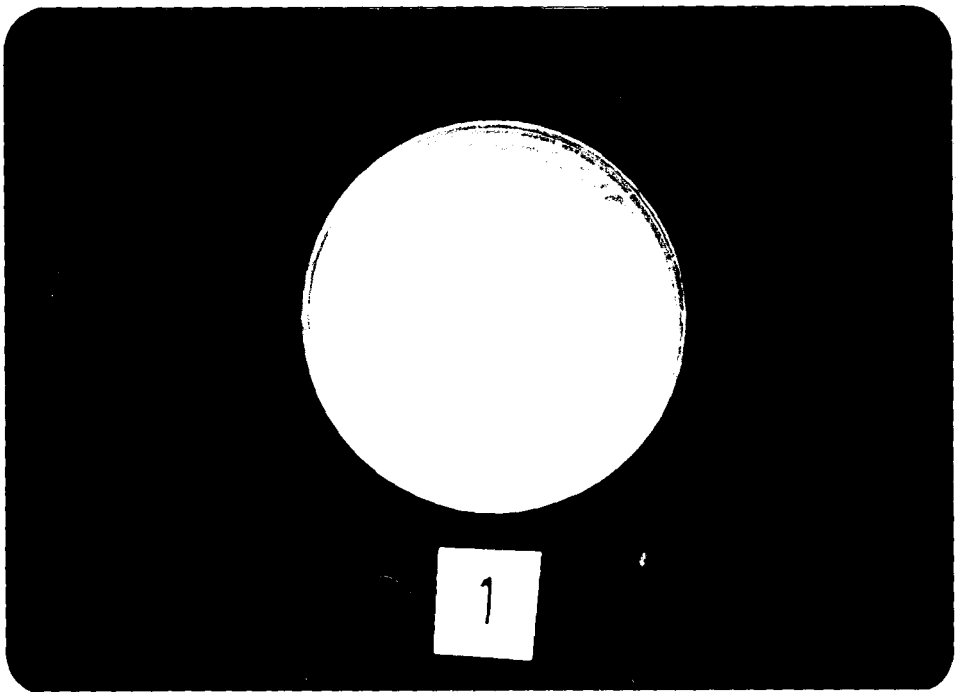
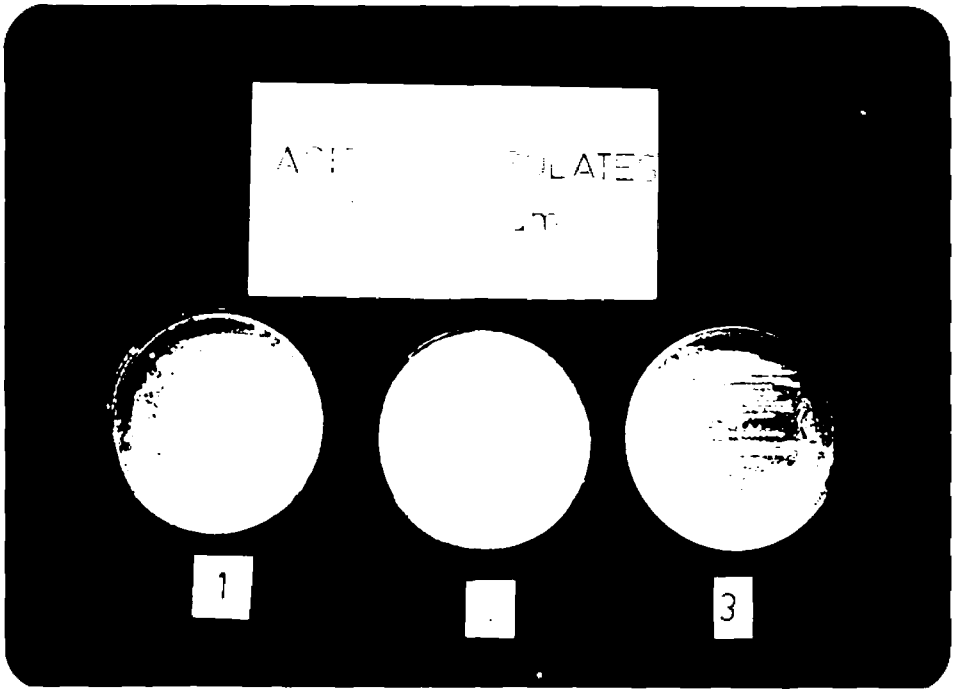


PLATE : iii



ENLARGED VIEW OF
AZOSPIRILLUM ISOLATE 3
WHEN STREAKED IN AGAR PLATES

PLATE : I



ISOLATES OF AZOSPIRILLUM IN AGAR PLATES

PLATE : II

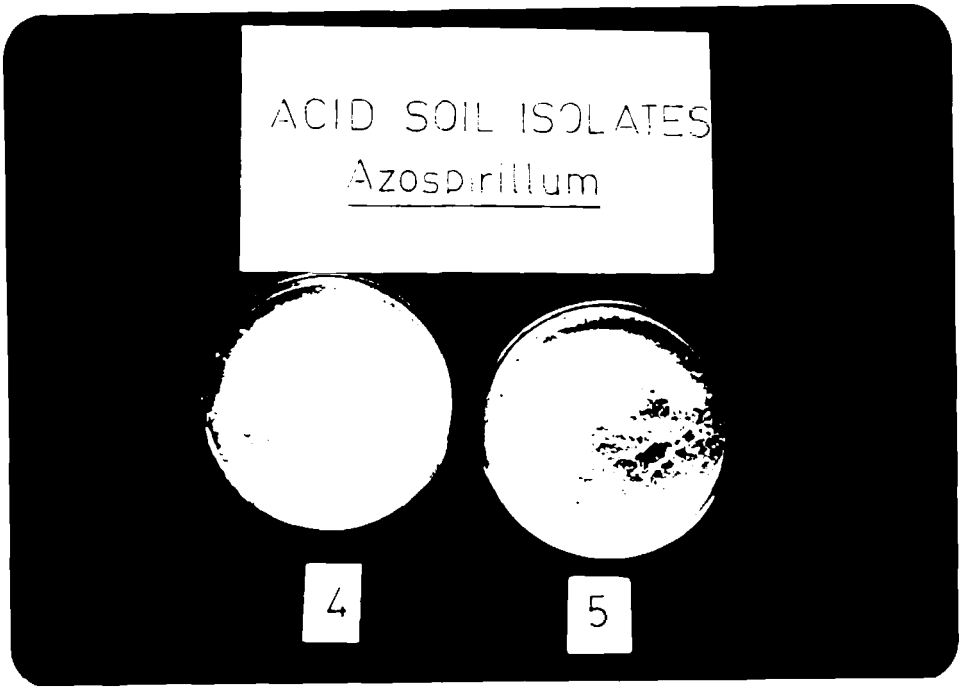
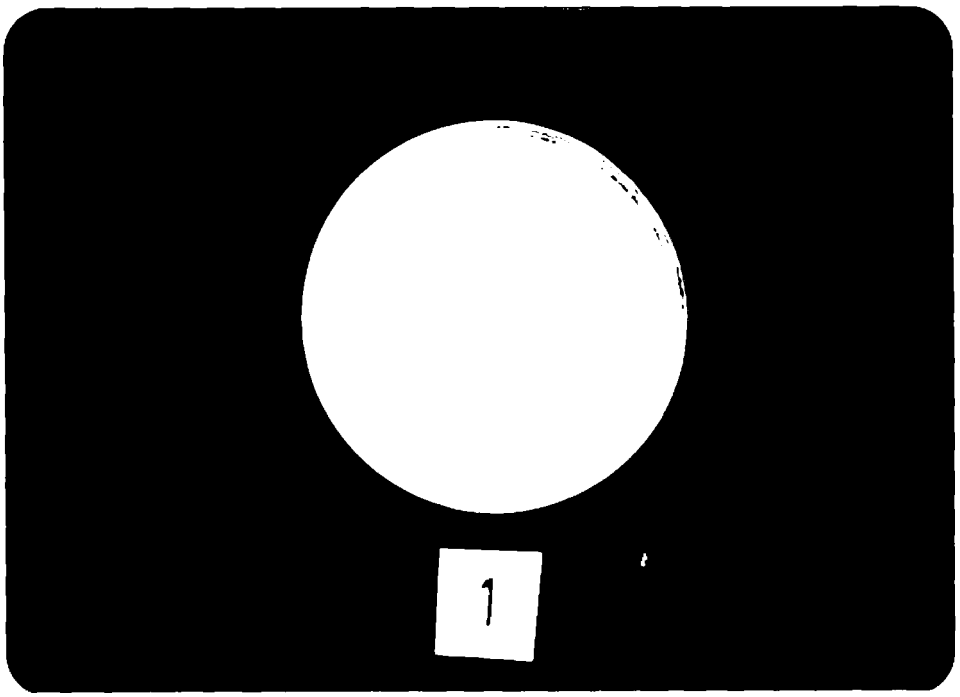


PLATE : iii



ENLARGED VIEW OF

AZOSPIRILLUM ISOLATE 3

WHEN STREAKED IN AGAR PLATES

CHARACTERISATION OF AZOSPRILLUM FROM ACID SOILS.

Azospirillum, the soil bacterium, widely distributed in almost all types of soils, had been isolated easily from all types of soils. Problems of the soil like salinity, alkalinity and acidity are known to influence the microbial population and activity. Alkaline soils collected from various parts of Tamil Nadu counted a very low population Azospirillum and most of the diazotrophs were almost absent (Purushothaman et al, 1985). However inoculation experiments conducted in Florida with Azospirillum brasilense to slightly acidic soils showed increased acetylene reduction (Smith et al 1984). Combined inoculation of Azospirillum and Azotobacter to Bajra in acidic soils showed increased crop and fodder yield (Pant et al, 1985). The population of Azospirillum in the acidic soils collected from Kuttanadu of Kerala State was very high when compared to the total heterotropic bacterial (14 to 15 x 10^8) population and diazotrops. (8 x 9 x 10^4). The population was found to be 0.15 percent of the total heterotrops on an average in these soils. Acidic soils thus have shown surprisingly a good population of Azospirillum inspite of the low counts of diazotrophs and lethal effects of the soil due to acidity. Pot culture and field trials of the bacterium gave better response than Standard Azospirillum brasilense strain sp.7 (Purushothaman et al, 1985).

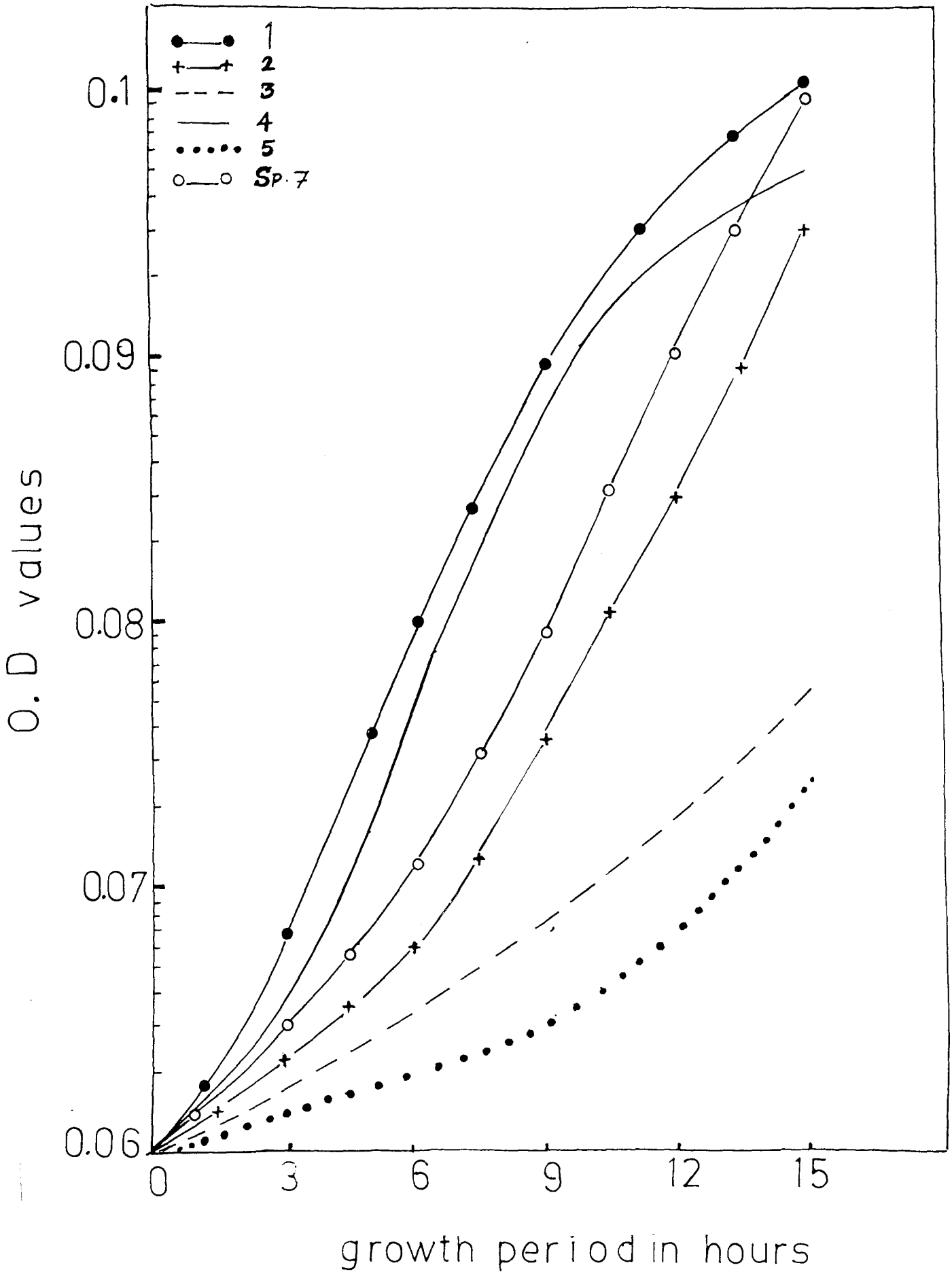

Growth of Azospirillum isolates
1
2
3
4
5
Sp. 7
0.1
0.09
0.08
0.07
0.06
O. D values
0
3
6
9
12
15
growth period in hours

FIGURE: 1

Azospirillum was isolated from sorghum roots grown in the soil collected from Kerala State. All the isolates were characterised following the traits laid down by tarrand et al, (1978). All the isolates were Azospirillum lipoferum spp. Majority of the isolates were found to exhibit both nitrate (nar^+) reductase and nitrite reductase (nir^+) activity as reviewed by Dobereiner et al, (1981). All these acid soil isolates were found to grow well in acidic environment as compared to the standard isolate of Azospirillum brasilense (Sp.7) from Brazil. [PLATE I, II, III]

GROWTH RATE OF AZOSPIRILLUM ISOLATES

TABLE III

Isolates of Azospirillum	3rd hr.	6th hr.	9th hr.	12th hr.	15th hr.	18th hr.
1	0.067	0.081	0.089	0.094	0.101	0.11
2	0.062	0.066	0.074	0.083	0.093	0.101
3	0.060	0.063	0.067	0.072	0.076	0.082
4	0.064	0.076	0.085	0.093	0.095	0.102
5	0.061	0.062	0.066	0.068	0.072	0.08
SP7*	0.063	0.070	0.079	0.090	0.099	0.103

* Optical density values of the isolates measured at 520 nm Isolates grown in yeast extract glucose medium of pH 5.0.

* Sp 7: International standard, A. Brasilense

Growth pattern of 5 isolates of Azospirillum are presented in figure I. Table II also represents the optical density values of the isolates. All the isolates were found to grow extremely well in the yeast extract glucose medium adjusted to pH 5.0. The standard isolate Sp 7 also recorded a high growth rate.

Isolates 3 and 5 recorded a slower growth than the other isolates. Within 3 hours all the isolates, reached the initial logrithamic phase which prolonged for approximately 15 hours for all the isolates.

CELL YIELD OF AZOSPIRILLUM IN YEAST EXTRACT GLUCOSE MEDIUM.

TABLE IV

ISOLATES OF AZOSPIRILLUM	CELL YIELD*
1	20
2	25
3	25
4	20
5	20
Sp 7	20

* In gm per litre of the cultuse medium

The cell yield of the Azospirillum isolates grown in a rich medium (yeast extract glucose medium) is presented in Table IV. A quantity of approximately 20-25 gram of dry weight of the cells per litre was harvested in a period of five days of all the isolates. Isolates 2 and 3 recorded maximum growth yield.

The high cell yield recorded by isolates 2 and 3 was useful in testing these two isolates at various pH and other experimental conditions and field trails.

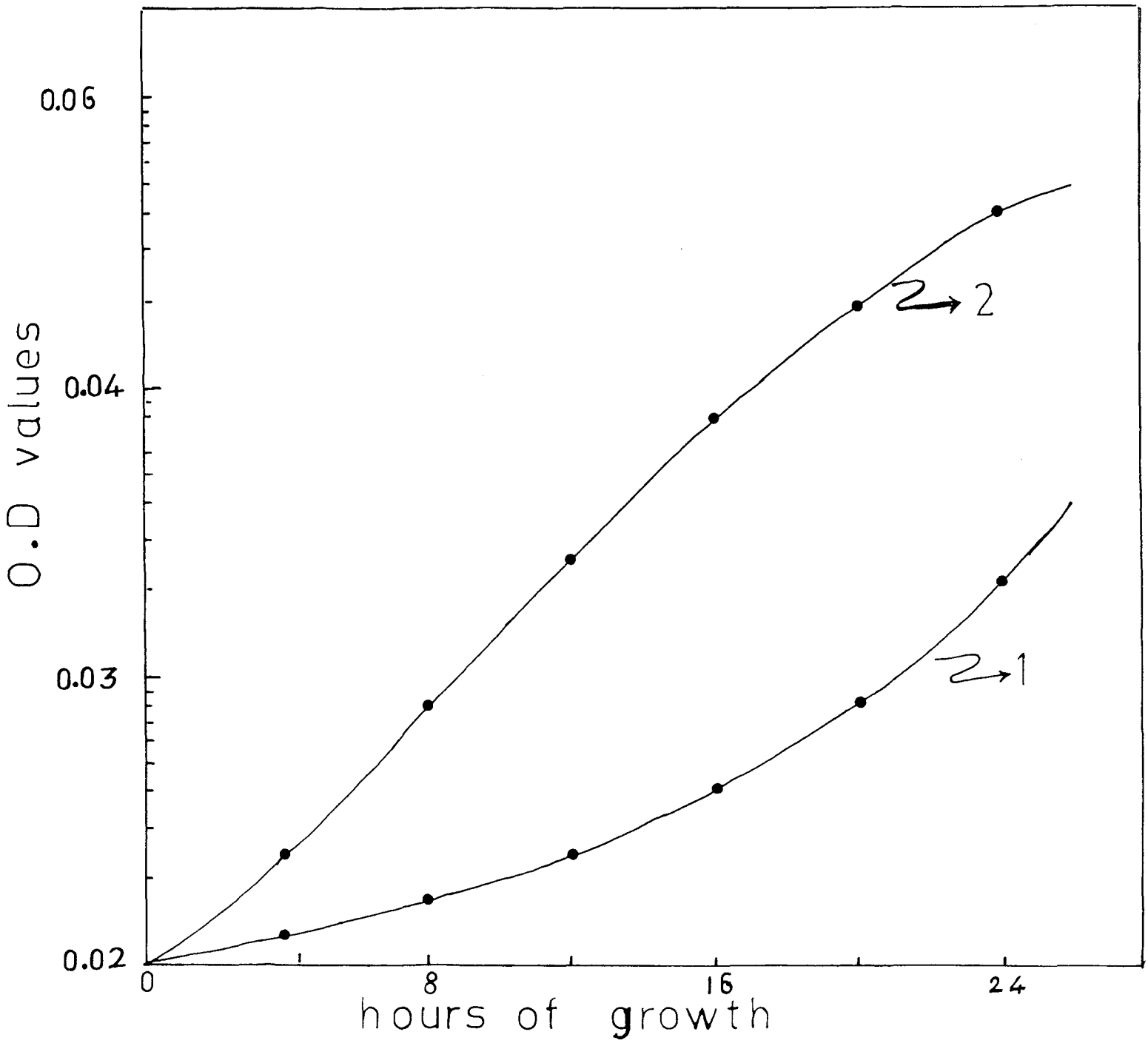

Growth of Azospirillum at pH 4.0
0.06
0.04
0.03
0.02
O.D values
2
1
0
8
16
24
hours of growth

FIGURE: 1

EFFECT OF PH ON AZOSPIRILLUM ISOLATES

TABLE V

Isolates of Azospirillum	OPTICAL DENSITY*						
	0th hour	4th hour	8th hour	12th hour	16th hour	20th hour	24th hour
1	-	0.020	0.021	0.022	0.024	0.028	0.032
2	-	0.022	0.028	0.033	0.038	0.042	0.045

* Optical Density measured at 520 nm

PH 4.0

Two of the 5 isolates, namely isolate 2 and 3 were grown in yeast extract glucose medium adjusted to pH 4.0 after sterilisation. Figure II represents the growth of these two isolates at pH 4.0. It was interesting to observe from the figures, the profuse and rapid growth of isolate 2 at pH 4.0 while the isolate 3 grew rather slowly with extended lag phase. These data indicated although the isolates were obtained from soils with pH 5.2, they are capable of thriving even at lower pH values. The optical density valuesused in the determination of growth rates of the isolates are represented in table IV.

EFFECT OF PH ON AZOSPIRILLUM ISOLATES

Azospirillum isolates grown in liquid yeast extract glucose medium of pH 5.0 showed a high growth rate as compared to the standard sp.7 (Fig.I). All the isolates reached the initial lograthamicphase within three hours and the growth phase of the isolates were complete by twenty four hours. The isolates through, this invitro experiment proved the capacity of the bacterium Azospirillum to grow in varied physical environments. These isolates were also grown at PH of 4.0, Though only two of the isolates were tested both the isolates surprisingly grew well at the low acidic pH even though the initial lag phase was slightly longer as compared to the same isolates grown at PH 5.0.

Growth of the isolates at PH 4.0 proved the ubiquitious nature of Azospirillum. Most of the diazotrophs failedto grow at this range of pH. The capacity of the isolates to thrive at very low pH, though they have been isolated initially from a PH range of 5.2 to 5.5 gives way for the application of this bacterium to even highly acidic soils. Most of the isolates obtained from similar soils were found to be of A.Lipoferum and all the isolates were found to grow well at acidic ph ranging from pH 4.0 to 6.0 (Kosslak et al, 1984). In vitro experimental results of Azospirillum at liquid culture medium of various pH, had thus led to the further study of Azospirillum in various acidic tropical soils.

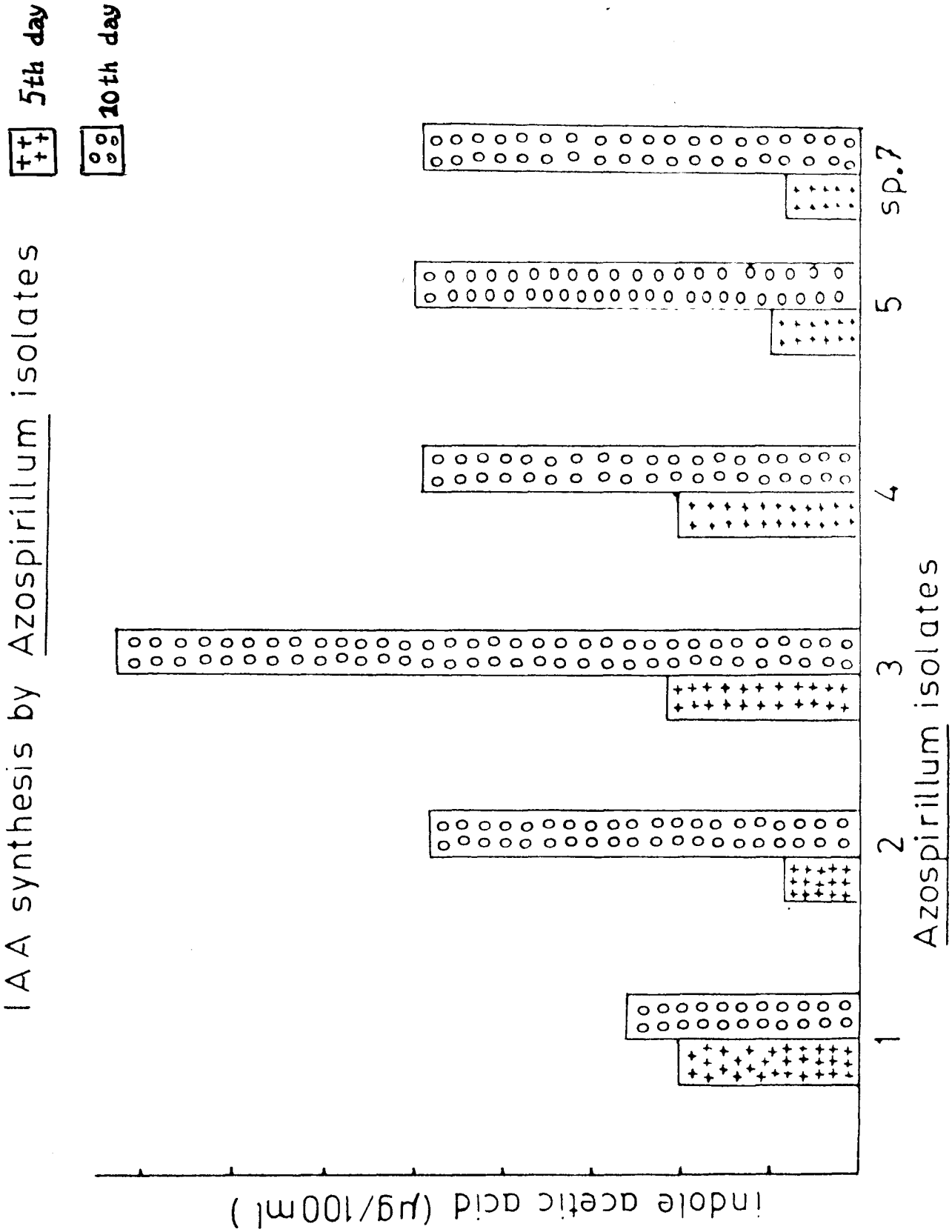

IAA synthesis by Azospirillum isolates
5th day
10th day
Indole acetic acid (µg/100ml)
1
2
3
4
5
sp.7
Azospirillum isolates

FIGURE: III

TOTAL INDOLE ACETIC ACID PRODUCTION BY AZOSPIRILLUM ISOLATES

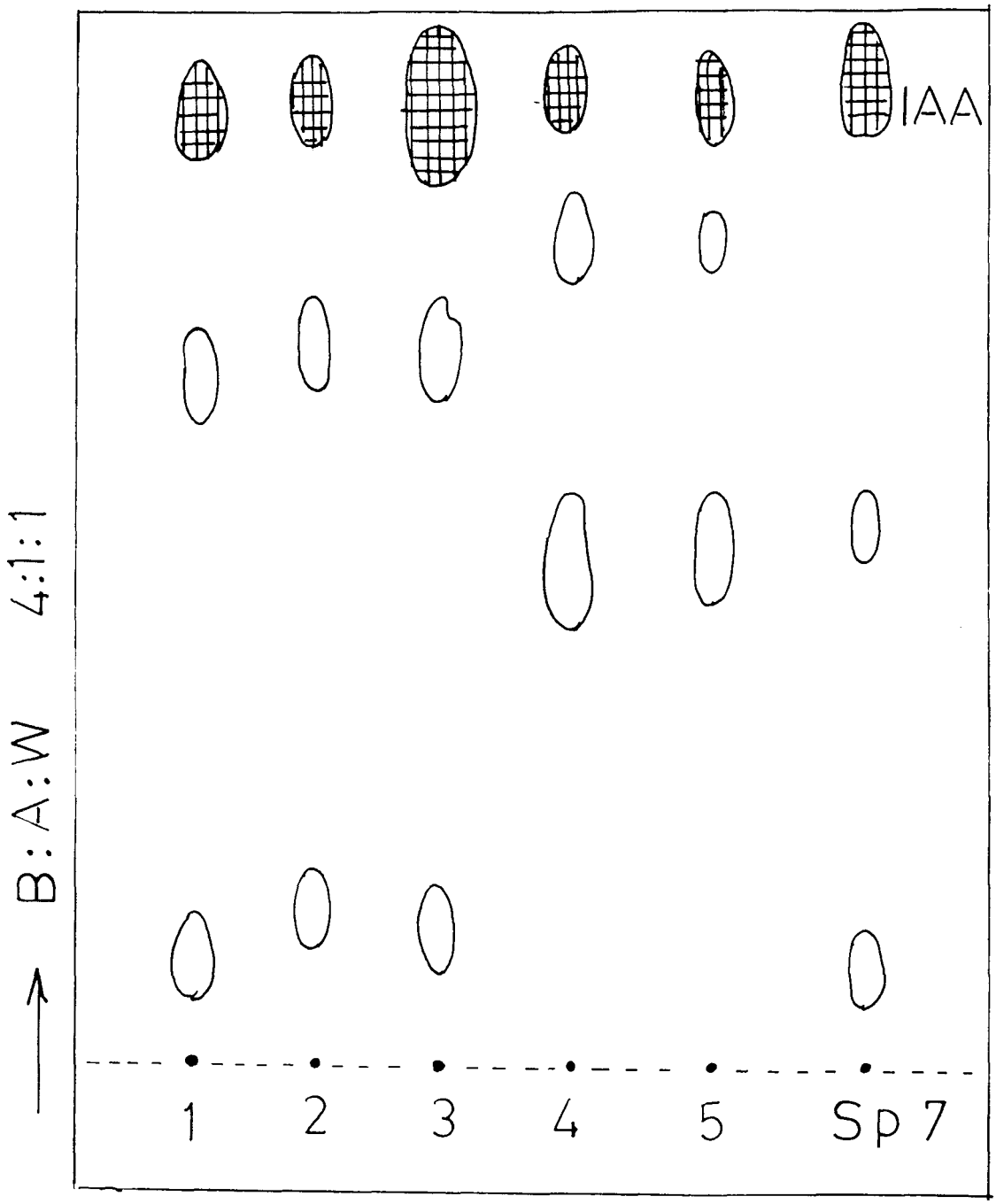
TABLE VI

ISOLATES OF AZOSPIRILLUM	QUANTITY OF INDOLE ACETIC ACID *	
	5th day	10th day
1	81.048	117.89
2	33.456	213.672
3	88.416	331.56
4	81.048	176.83
5	36.84	180.49
SP7	33.416	176.83

* Indole acetic acid formed in microgram per 100 ml of culture medium under (-) tryptophan condition medium not supplemented with tryptophan.

The capacity of the isolates of Azospirillum for synthesising phytohormones like Indole acetic acid (IAA) are presented in Table VI. The growth medium was essentially yeast extract glucose broth not supplemented with the precussor tryptophan*. The total indole acetic acid in the cell free culture filter was determined at two growth period. This data is represented in the form of a histogram in figure III. These data indicated that even without tryptophan the isolates synthesised fairly large quantities of indole acetic acid (normally 150 to 360 microgram). On fifth day isolates 3 and 4 registered high values of indole acetic acid while on tenth day isolates 3 and 2 recorded maximum amount of indole acetic acid. In this respect all the acid soil isolates of Azospirillum were superior to isolate Sp.7.

Chromatogram



Indole compounds in the cell-free culture filtrate.

FIGURE: IV

*Tryptophan is the precuesor of indole acetic acid production IAA production was found to be 150 to 360 microgram per 100ml of culture medium supplemented with tryptophan.

CHROMATOGRAPHIC IDENTIFICATION OF *INDOLE* ACETIC ACID AND INDOLE COMPONENTS OF AZOSPIRILLUM ISOLATES

TABLE VII

ISOLATES OF AZOSPIRILLUM	RF VALUES
1	0.107 0.653
2	0.147 0.693
3	0.133 0.680
4	0.480 0.780
5	0.587 0.787
SP7	0.937 0.507
STANDARD IAA	0.913

The occurence of indole acetic acide and other indole derivatives in the culture filterate of the isolates are set out in the chromatogram in Figure IV. Along with indole acetic acid a few more indole derivatives were also recorded on the chromatogram. All the five isolates and the standard sp 7 recorded more than one indole compound. The RF values of the indole compounds and indole acetic acid are given in Table.VI.

PHYTOHORMONE PRODUCTION BY AZOSPIRILLUM

Azospirillum isolates were also found to produce significant amount of phyto hormones like indole acetic acid. Most of the isolates produced indole acetic acid in significantly large amounts as compared (170 to 300 microgram per ml) to the standard sp.7. Indole acetic acid synthesis was found to increase in most of the Azospirillum strains as the nitrogen fixing capacity of the strains increased (Hartman et al 1983). Tests carried out on the effect of plant growth promoting substances like gibbevlins and indole acetic acid on plant, growth, showed increased plant growth and plant biomass as the production of growth, promoting substances increased (Silveria et al, 1983). The bacterial growth was found to increase in medium supplemented with tryptophan, the precussor for the production of indole compounds (Moore, 1980) - The indole acetic acid concetration was found to have a linear effect on the growth of plants and bacterial population.

NITROGEN FIXING CAPACITY OF THE ISOLATES

Nitrogen fixing capacity of the isolates, varied between isolates and this hasled a way to inoculate these isolates to various plants. Nitrogen fixing capacity of Azospirillum strains inoculated to rice soils showed enhanced acetylene reduction activity at low nitrogen content (Nayak et al, 1981). Nitrogen fixing capacity of the Azospirillum strains in various non leguminous plants like wheat (Avivi et al 1982) maize (mellado et al 1983) Setaria italca (Okon et al 1983),

Balrey and maize (Kulinka et al 1983) showed active acetylene reduction and varied nitrogen fixing capacity.

NITROGENASE ACTIVITY IN AZOSPIRILLUM ISOLATES

TABLE VIII

ISOLATES OF AZOSPIRILLUM	NITROGENASE ACTIVITY *
1	60
2	70
3	70
4	70
5	50
Sp 7	50

* nanomoles of ethylene produced per hour per mg of protein.

The nitrogen fixing capacity of the isolates of Azospirillum was studied in a gas liquid chromatogram. The data of nitrogenase activity are presented in Table VIII. The nitrogenase activity (ARA) of acid soil isolates and SP 7 were anywhere between 50 to 70 n moles of ethylene per mg of protein per hour. Isolates 2, and 4 showed maximum nitrogenase activity while the nitrogenase activity of isolate 5 was found to be minimum.

Nitrogenase enzyme system is the metallo enzyme complex present in all nitrogen fixing micro-organisms. Nitrogenase enzyme system helps the bacteria to fix the elemental atmosphereic nitrogen as ammonia in the soil. The nitrogenase activity of the isolates was found to be low when compared to the normal activity (300 to 400 n moles) in all

PLATE : IV

CULTURES OF AZOSPIRILLUM FOR NITROGENASE ACTIVITY

Azospirillum species at neutral pH, inspite of low acetylene reduction activity, the nitrogenase action and the nitrogen fixing capacity of the acid soil isolates at pH 5.0 was sufficient to increase the plant vigour index in peat based soil in this study.

NITRITE REDUCTASE ACTIVITY IN AZOSPIRILLUM ISOLATES.

ISOLATES OF AZOSPIRILLUM	NITRITE REDUCTASE ACTIVITY*	
	THIRD DAY	FIFTH DAY
1	13.453	14.08
2	14.08	14.39
3	4.29	5.32
4	0.765	1.53
5	1.23	2.76
SP7	14.39	14.54

* Micromoles of Nitrite present in one ml of culture medium

Nitrite redutase activity in Azospirillum isolates are shown in Table IX. All the isolates possessed nitrite reductase (nir +) activity. Isolates 3 and 4 recorded maximum of nitrite reductase activity. Sp 7 and isolate I registered low nitrite reductase activity. The nitrite reductase activity was measured at two growth periods.

Nitrite reductase activity indicates the dentrification property of Azospirillium Nitrite reductase oxidies, the ammonia fixed by the bacterium to nitrite. Thus ammonia unavailability found to plants was reduced. Denitnifrication was only found in

mutants of Azospirillum in the range of 1.2 to 2.5 micro miles of nitrite per ml in a nitrite grown medium (Dobereiner, 1982). All the acid soil isolates showed high denitrification in a nitrite grown medium except isolate 2 and 1.

NITRATE REDUCTASE ACTIVITY IN AZOSPIRILLUM ISOLATES

TABLE X

AZOSPIRILLUM ISOLATES	NITRATE REDUCTASE ACTIVITY *	
	3rdday	5th day
1	1.86	1.24
2	4.35	1.86
3	2.48	1.86
4	0.621	Nil
5	3.72	2.48
sp7	11.8	11.43

* Quantity of nitrate present in micromoles per ml of culture medium.

Nitrate reductase (nr) activity of the isolates of Azopirillum is presented in Table X. All the six isolate were positve of nitrate reductase (nr +). The isolate sp 7 recorded the highest nitrate reductase activity than the rest of the isolates. Isolate 4 surprisingly showed very low nitrate reductase activity than other isolates.

Nitrate reductase, the second denitrifying enzyme converts nitrite formed from ammonia to nitrate and further to nitric oxide. Nitric oxide might escape into the atmosphere, thus completing the nitrogen cycle. The above

data has clearly indicated that the nitrate reductase activity of the isolates was low in a nitrite supplemented medium when compared to the wild type strain of Azospirillum brasilense (9 to 12 micromoles of nitrite per ml). The combined effect of the two enzymes might inhibit the nitrogenase activity by utilizing the ammonia that was fixed by the bacteria.

QUANTITY OF AMMONIA EXCRETED BY ISOLATES OF AZOSPIRILLUM

TABLE XI

AZOSPIRILLUM ISOLATES.	QUANTITY OF AMMONIA*		
	7th day	10th day	15th day
1	3.8	6.72	12.48
2	1.9	3.36	11.04
3	1.92	4.32	15.76
4	4.32	5.28	6.0
5	1.92	2.88	13.2
sp 7	0.48	3.34	9.6

* quantity of ammonia present in 100 ml of the culture medium in milligram i.e. mg/100ml.

The data on the release of fixed ammonia from the nitrogen fixing cells of Azosprillum isolates are set out in Table XI. All the isolates showed the excretory property of ammonia. All the five isolates were found to excrete more ammonia than that of the standard sp.7. The isolate 3, 5 and 1 were found to record higher quantities of ammonia while the isolate 4 however recorded a very low value of ammonia compared to all the isolates.

The capacity of the Azospirillum isolates to excrete the fixed ammonia into the medium was found to be beneficial to plants. This reveals the property of the bacterium to make available the nitrogen fixed in the form of ammonia in the rhizosphere of the bacterium, so that the plants might absorb the nitrogen. The Azospirillum strains were found to have a low nitrate reductase activity in both the nitrite grown and nitrate grown medium. Mutants of A.lipoferum were only found to have this property (Purushothaman et al, 1984). This denitrification property of the isolates may inhibit the nitrogenase activity of the isolates in the medium. However even with this nitrate reducease activity, the isolates were found to have a good nitrogen fixing capacity. Due to this property of the isolates to extrete the ammonia fixed in the rhizosphere into the environment, the nitrogenase action was not inhibited.

INFLUENCE OF AZOSPIRILLUM INOCULATION TO PLANT GROWTH:

The need of nitrogen to the growth of the plants have lead to the inoculation of the crops with Azospirillum (Kapulnik, 1984, Tilak, 1985, Okon et al., 1983, Vose, 1983). Several scientists have proved an increased crop yield after inoculation of the plants with Azospirillum (Baldani et al, 1983). Thus inoculation of Azospirillum to various crops in various soils also showed an increased growth of the plants (Kundu et al, 1983).

In this study, the Azospirillum strains, isolated from the acid soil samples were inoculated to maize and sorghum similar characteristics as the soil samples collected from Kuttanad of Kerala State. The results of the pot culture experiments on the influence of Azospirillum inoculation to sorghum and maize and presented in Table XII and XIII.

INFLUENCE OF AZOSPIRILLUM INOCULATION ON PLANT GROWTH (SORGHUM BICOLOUR VAR. CO. 24)

TABLE XII

AZOSPIRILLUM ISOLATES	SHOOT LENGTH (CM)	ROOT LENGTH (CM)	FRESH WEIGHT (g)	DRY WEIGHT (g)
1	19	20.2	0.48	0.083
2	18.6	19.4	0.41	0.083
3	22	20.1	0.61	0.11
4	16.2	16	0.41	0.072
5	18.0	17.5	0.51	0.09
sp 7	19.3	14	0.51	0.088
INOCULATED	16	12.3	0.40	0.070

* 15 days of plant growth, Mean of four plants. Plants grown in peat soil.

	ROOT LENGTH cm	SHOOT LENGTH cm	FRESH WEIGHT g	DAY WEIGHT g
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
INOCULATED	19.1 ± 2.01	18.0 ±0.204	0.49 ±0.4	0.086±0.006
UNINOCULATED	16 ± 0.210	12.3 ±0.058	0.40±0.006	0.07 ± 0.005
t cal	24.78	2.623	6.998	9.009

$t_{0.05} = 2.06$, $V = 25$

$t_{0.01} = 2.79$, $V = 25$

V = Degree of freedom.

INFLUENCE OF AZOSPIRILLUM ISOLATE INOCULATION ON PLANT GROWTH (MAIZE) (Var-gauge-5).

TABLE XIII

ISOLATED OF AZOSPIRILLUM	ROOT LENGTH	SHOOT LENGTH	FRESH WEIGHT	DRY WEIGHT
	cm	cm	g	g
1	30.66	37.33	4.38	0.58
2	28.75	36.75	4.235	0.43
3	36	46	5.97	0.99
4	28.5	45	5.61	0.67
5	35.75	47	5.86	0.482
sp 7	24.5	80.5	2.88	0.6
UNINOCULATED	27	38	3.51	0.41

* Plants grown for 15 days, Mean of 4 plants, Plants grown in peat soil.

	ROOT LENGTH cm	SHOOT LENGTH cm	FRESH WEIGHT g	DRY WEIGHT g
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
INOCULATED	31.3 ± 0.619	42.1 ± 0.73	4.9 ± 0.58	0.58 ± 0.062
UNINOCULATED	27 ± 0.460	38 ± 0.5	3.51 ± 0.062	0.41 ± 0.01
t cal	21.78	77.64	14.86	4.47

t 0.05 = 2.06 v = 21

t 0.01 = 2.83 v = 21.

PLATE V

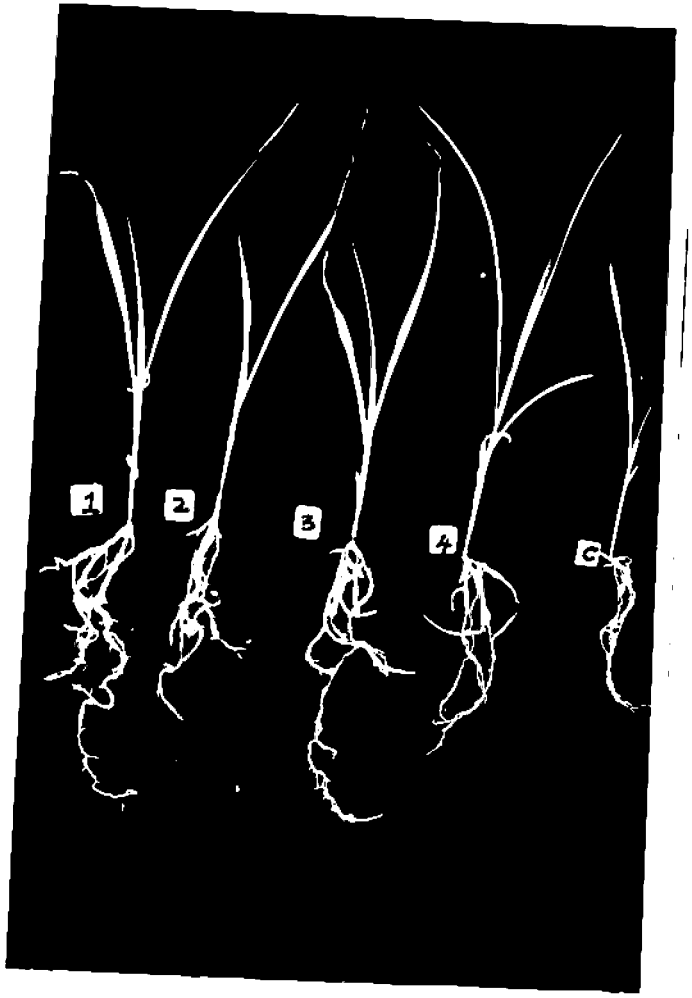
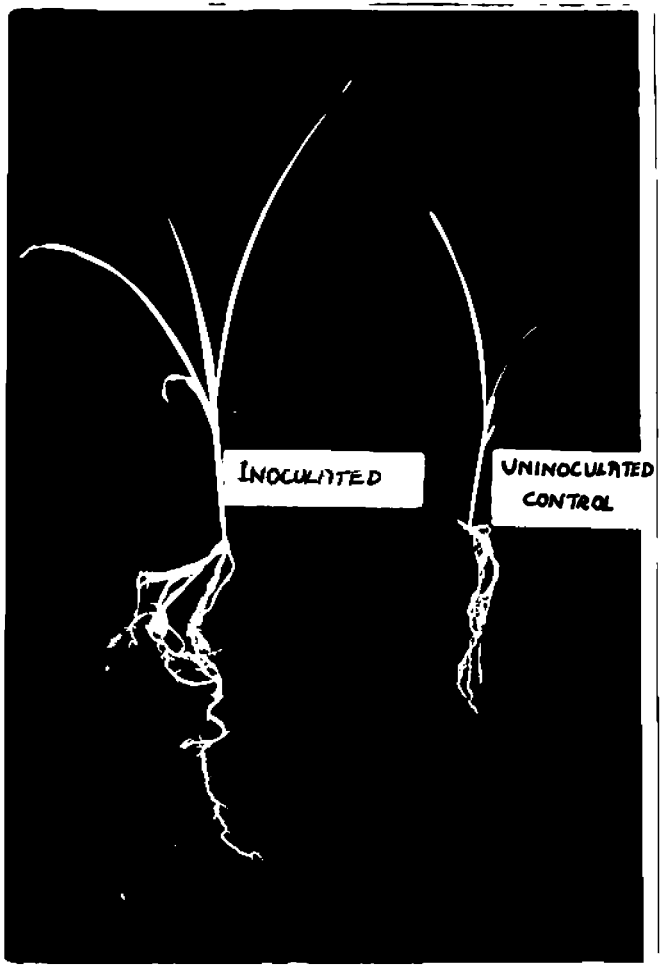


PLATE VI



INFLUENCE OF AZOSPIRILLUM INOCULATION ON PLANT GRONTH [SORGHUM Va. Co. 24]

The results of the pot culture experiments on the influence of Azospirillum inoculation in Sorghum and maize are presented in Table XII and XIII. Seed and soil inoculation of Azospirillum for sorghum resulted in a significant increase in shoot length, root length, fresh weight of the plant and the dry weight of the plant. Similarly in maize crop also a signficant increase in the root length, shoot length, fresh weight and dry weight of the plant were encountered due to Azospirillum inoculation. It is to reconside that the soil used in these experiments were having a pH of 5.2 to 5.4 as it was a peat mixture soil.

Thus the inoculation studies have indicated a significant increase in the plant root length, shoot length fresh weight and dry weight of the plants when compared with the uninoculated control and also, the standard Azospirillum brasilense strain (sp 7). There was a significant increase in root biomass within a period of twenty days of plant growth. There was significant increase in root length and also in the shoot length of the plants when compared with the uninoculated controls. Thus these results clearly indicates the effectiveness of Azospirillum inoculation on plants growth. Thus inoculation of Azospirillum to peat based soil was found to show an over all increases in all growth parameters of and vigor index of the plants. From all the above results it is clear that of the 5 isolate, isolate 3 was found to be very effective. Isolate 3 was found to produce maximum IAA, and also showed high nigrogenase activity and ammonia excretion. The response of sorghum and maize to Azospirillum isolate 3 was found to be very effective compared to all the other isolates.

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Summary and Conclusion

SUMMARY AND CONCLUSION

A study was undertaken to characterise the associative nitrogen fixing bacterium *Azospirillum* in acid soils collected from Kuttanad and Trichur of Kerala State. The soils samples collected were tested for total hetrotrophs, diazotrophs, and *Azospirillum*. The study was conducted in two phases.

First phase of the experiment consisted of isolating the bacteria *Azospirillum* from sorghum roots grown in the acid soils collected from Kerala State. The isolated *Azospirillum* species were than characterised according to the traits laid down by Tarrand *et al* (1978).

In the second phase of the study, the isolates of *Azospirillum* were inoculated to various medium in *invitro* conditions and to certain pot cultures. Experiments conducted revealed the growth pattern of the *Azospirillum* at various pH, nitrification and denitrification property of the isolates, Ammonia excretion of the isolates and also the production of phytohormones by the isolates. The effect of *Azospirillum* inoculation on the earth growth of sorghum and maize under pot culture condition were also studied. The results of the study are summarised as follows.

The indole compounds identified and indole acitic acid produced were found to increase the plant growth.A linear relation was found between the amount of IAA produced and the effect of the inoculation of the isolates to various crops.

9) The effect of inoculation of the Azospirillum isolates to sorghum and maize on peat based soil were studied. It was found that there was a significant increase in the root length, shoot length, plant fresh weight and dry weight. There was in overall increase in the plant weight as compared to the controls and standard. This experiment on the peat based soil of pH 5.0 proved that Azospirillum inoculation was effective to plants grown in acidic soils.

In general, we can say that Azospirillum lipoferum was very effective in increasing the vigour index of the plants grown in acidic conditions. The Azospirillum could thus be inoculated to tropical acidic soils effectively. This diazotroph was found to fix nitrogen in all types of habitats. Thus Azospirillum was proved to be a very effective diazotroph when compared to the existing nitrogen fixing capacity of this associative bacterium with respect to cereals and grasses clearly indicated that nitrogen fixation in non-legumes by Azospirillum occurs at level of agronomic significance in crops production in semi-arid tropics (Wani 1985).

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Recommendations

RECOMMENDATIONS

Azospirillum which has been proved to be bio fertilizer for varied habitats can be recommended as a bio-fertilizer to acid soils, though it cannot completely supplement the chemical fertilizer needof the soil.

Azospirillum could be inoculated as a nitrogen fixer to maize, sorghum, wheat other grasses and non leguminous plants that grow in acidic soil fields for further trail experiments and the effectiveness could be tested over a long duration.

Experiments on influence of the soil physiological properties like temperature, moisture content and pH on the nitrogen fixing capacity of the isolates could be conducted to increase the application of these isolates to even highly acidic soils. Further study on the dentrifying capacity of the isolates can be carried out to reduce the activity of the enzymes so that *Azospirillum* inoculation might be highly effective.

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