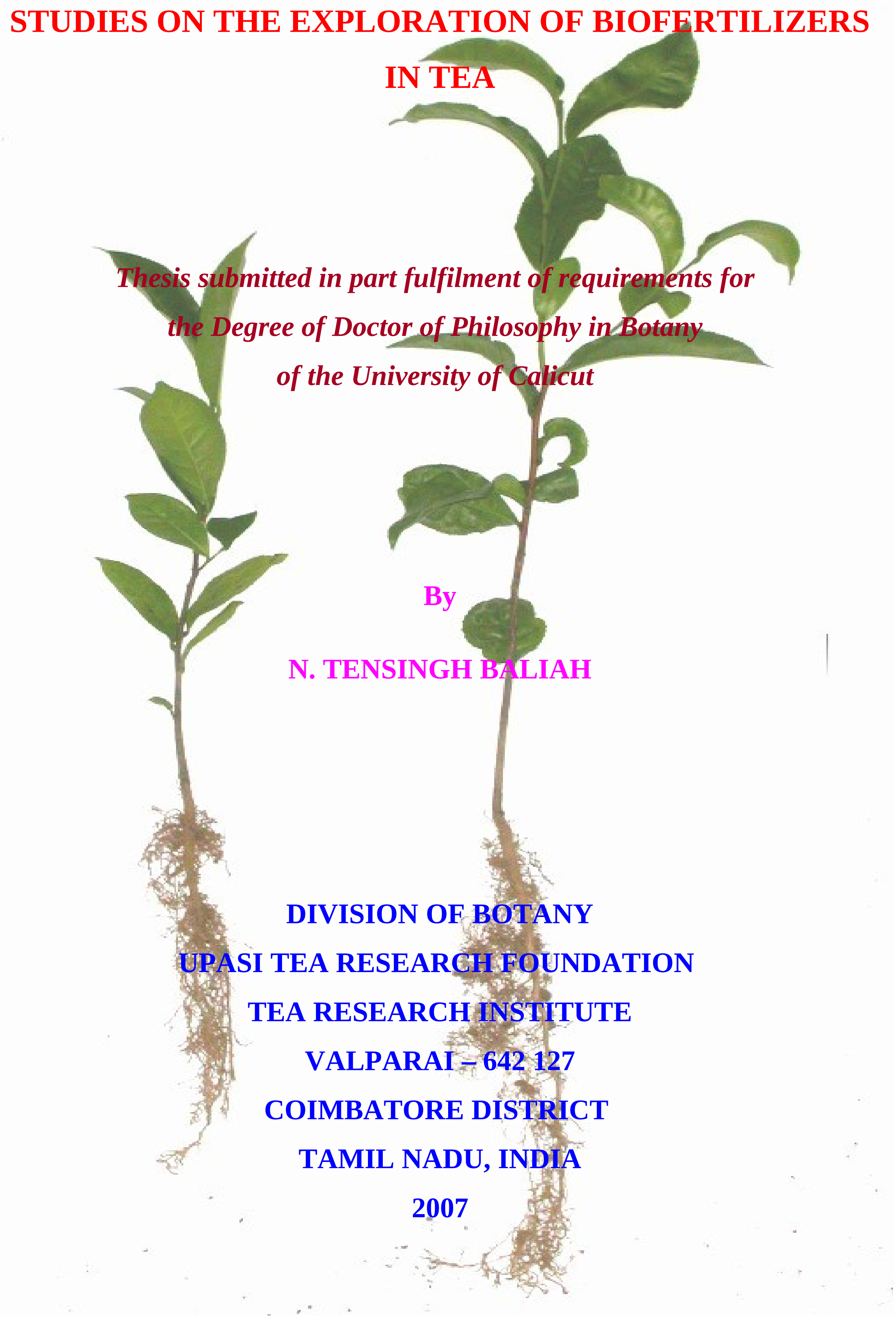

STUDIES ON THE EXPLORATION OF BIOFERTILIZERS IN TEA

Thesis submitted in part fulfilment of requirements for
the Degree of Doctor of Philosophy in Botany
of the University of Calicut

By

N. TENSINGH BALIAH

DIVISION OF BOTANY
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VALPARAI – 642 127
COIMBATORE DISTRICT
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Dr. U.I. Baby
Research Guide

CERTIFICATE

Certified that this thesis entitled "**STUDIES ON THE EXPLORATION OF BIOFERTILIZERS IN TEA**" embodies the results of a piece of bonafide research work carried out as part of fulfilment of requirements for the degree of Doctor of Philosophy in Botany of University of Calicut by **Mr. N. Tensingh Baliah** under my guidance and supervision and that no part of the thesis has been submitted for any other degree.

I further certify that all helps and sources of information availed of in this connection have been acknowledged.

Valparai **(Dr. U.I. BABY)**

DECLARATION

I, **N. Tensingh Baliah** hereby declare that the thesis entitled **“STUDIES ON THE EXPLORATION OF BIOFERTILIZERS IN TEA”** being submitted in partial fulfilment of the requirements of Ph.D. Degree in Botany of University of Calicut embodies the results of a bonafide work done by me under the guidance of **Dr. U.I. Baby, M.Sc., Ph.D**., UPASI Tea Research Foundation, Tea Research Institute, Valparai and that no part of it has been submitted for any other degree.

Valparai **(N.TENSINGH BALIAH)**

DEDICATED

TO

MY LORD

PARENTS

&

TEACHERS

ACKNOWLEDGMENT

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Valparai **N. Tensingh Baliah**

ABBREVIATIONS USED IN THESIS

$AlPO_4$	- Aluminium phosphate
AFLP	- Amplified Fragment Length Polymorphism
ARA	- Acetylene Reduction Assay
Azo	- *Azospirillum*
bp	- base pair
BSS -1	- Biclonal Seed Stock - 1
cm	- centimeter
C_2H_2	- Acetylene
C_2H_4	- Ethylene
C_2H_5	- Acetylene
$CaCl_2$	- calcium chloride
$Ca_3(PO_4)_2$	- Tricalcium phosphate
CD	- Critical Difference
cfu	- colony forming unit
COC	- Copper Oxy Chloride
CO_2	- carbon di oxide
CuO_2	- copper oxide
DNA	- Deoxyribo Nucleic Acid
dNTPs	- deoxy Nucleotide Triphospahtes
EC	- Electrical Conductivity
EPS	- Exopoly saccharide
Fe	- Iron
Fe^{3+}	- ferric
Fe_2O_3	- Ferric oxide
$FePO_4$	- Ferric phosphate
Fe_2PO_3	- Ferric phosphate
Fig.	- Figure
FYM	- Farm Yard Manure
FID	- Flame Ionizing Detector
g	- gram

GA3	- Gibberellic Acid
GR	- Graph Reading
h	- hour (s)
H_2O_2	- hydrogen per oxide
ha	- hectare
HCl	- hydrochloric acid
IAA	- Indole Acetic Acid
K	- potassium
kg	- killo gram
KH_2PO_4	- potassium di hydrogen phosphate
$KMnO_4$	- potassium permanganate
KNO_3	- potassium nitrate
LB	- Luria Bertaini
M	- molar
mg	- milli gram
$Mg_3(PO_4)_2$	- magnesium phosphate
min	- minutes
ml	- milliliter
mm	- milli meter
mM	- milli molar
MnO_2	- manganes oxide
Mo	- Molybdenum
MOP	- Muriate of Potash
n mole	- nano mole
N	- nitrogen
N	- normality
NaCl	- sodium chloride
NaOH	- Sodium hydroxide
NH_4	- ammonia
NO_3	- Nitrate
nm	- nano meter
nif gene	- nitrogen fixing gene

NRA	- Nitrate Reductase Activity
No.	- Number
OM	- Organic Matter
O_2	- oxygen
ºC	- degree Celsius
OD	- Optical Density
p mol	- pico mole
P	- phosphorus
PCR	- Polymerase Chain Reaction
pH	- negative logarithm of hydrogen ion concentration
PHB	- Poly -β-hydroxy Butyrate
ppm	- parts per million
PSB	- Phosphate Solubilizing Bacteria
PSM	- Phosphate Solubilizing microorganisms
P_2O_5	- Phosphorus penta oxide
RAPD	- Random Amplified Polymorphic DNA
rpm	- rotation per minute
SDS	- Sodium Dodecyl Sulphate
sp.	- species
spp.	- species (plural)
TBE	- Tris Borate Ethylene diaminetetra Acetic acid
TCP	- Tricalcium Phosphate
TEG	- Tris Ethylene diaminetetra acetic acid Glycine
TRF	- Tea Research Foundation
UP3	- UPASI - 3
UP9	- UPASI - 9
UP26	- UPASI - 26
UPASI	- United Planters’ Association of Southern India
USA	- United States of America
UV	- Ultra violet
VP	- Vegetative Propagated plants
Vit	- Vitamin

wt	- weight
µ mole	- micro mole
µg	- micro gram
µm	- micro meter
%	- per cent
@	- at the rate of
+ve	- positive
–ve	- negative
>	- greater than
<	- less than

PREFACE

Modern agriculture is aimed at increasing productivity through heavy inputs of chemical fertilizers and high yielding cultivars. Due to the heavy exploitation of the soil, the nutrient level is rapidly decreasing to an alarming level. Fertilizer application is one of the costly inputs in tea husbandry. Further, the ever increasing cost of chemical fertilizers, their limited supply and concern on environmental pollution due to the increased use of chemical fertilizers and pesticides increased interest in organic farming. It necessitates developing alternative strategies on nutrient management. Biofertilizers are important in this regard as they can supplement nutritionally important elements to the plant through biological processes. They are efficient, environmentally sound and help in sustainable productivity.

Use of biofertilizers like N-fixers, P-solubilizers and P-mobilizers will help the industry in rationalizing the fertilizer use and cut the cost of inputs. These biological resources can form an integral part of integrated nutrient management programme to improve soil health to sustain productivity.

The present study is a modest effort to isolate and screen the efficient nitrogen fixers and phosphate solubilizers from tea soil. The main objective of the study is to select region specific, acid tolerant strains of these organisms which can be effectively used in tea husbandry to increase the production and productivity.

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INTRODUCTION

Green revolution aimed at the increase in crop production through intensive cultural practices and use of improved high yielding cultivars. However, continuous use of chemical fertilizers resulted in the decline in organic matter content of the agricultural soil leading to depletion of beneficial microorganisms which inturn reduced the soil productivity. The agricultural land is diminishing exorbitantly and there is no scope for further expansion in area. Hence to meet the increasing demand, the crop production has to be increased per unit area of land. Man made fertilizers containing nitrogen, phosphorus and potassium, increased the output of agricultural products. The demand for chemical fertilizers is keep on increasing, widening the gap between the demand and supply. Such a gap would be difficult to bridge in the wake of the energy crisis. Therefore, the strategy for improving agricultural production in developing countries should consider supplementing nitrogen and phosphorus through microbial processes. This can be accomplished through the application of biofertilizers.

Biofertilizers are carrier based preparations containing beneficial microorganisms in a viable state. They improve soil fertility and promote plant growth. Biofertilizers are broadly classified into nitrogen fixers, phosphate solubilizers, phosphorus mobilizers and organic matter decomposers. They enhance certain biological processes by which the nutritionally important elements make available to the plants (Baby, 2002).

Nitrogen is the most important element required by the plants. Even though about 80 per cent of earth's atmosphere composed of nitrogen, plants cannot directly utilize it. Plants can absorb nitrogen only in the form of nitrate nitrogen and ammoniacal nitrogen. Soil heterotrophic bacteria like *Azospirillum* and *Azotobacter,* symbiotic bacteria like *Rhizobium* and certain blue green algae can readily utilize atmospheric nitrogen and convert it to absorbable form. Plants can readily utilize this assimilated nitrogen. These types of organisms are called nitrogen fixers.

Nitrogen fixers are either free living in soil or symbiotic with plants. Nitrogen fixers offer economically attractive and ecologically sound means of reducing chemical nitrogen inputs. The most important nitrogen fixers are *Azospirillum, Rhizobium, Azotobacter* and *Azolla*. Among these, the most promising organism capable of colonizing roots and exerting this miracle process is the bacterium belonging to the genus *Azospirillum*. *Azospirillum* is microaerophilic, gram negative and spiral in shape, which fixes atmospheric nitrogen asymbiotically. *Azospirillum brasilense, A. lipoferum, A. amazonense, A. halopraeferans, A. irakense* and *A. dodereinerae* are the different species of the genus. *Azospirillum* is present predominantly in acidic soil.

Phosphorus is another major plant nutrient required in optimum amount for proper plant growth. Phosphorus is known to involve in a plethora of functions in the plant growth and metabolism. The cellular machinery is difficult to be imaged without phosphorus being involved in its metabolic continuity and even perpetuation. Only about 25 per cent of the phosphorus applied to the soil is available for the crops and the rest become unavailable due to chemical fixation with aluminium and iron in acidic soils. Indian soils are characterized by poor and medium status with respect to available phosphorus.

There are various types of soil microbes which can solubilize this fixed form of P and make it available to plants. Such organisms are called phosphate solubilizers. Phosphate solubilizing microorganisms include bacteria, fungi and actinomycetes. Several soil bacteria, particularly those belonging to the genera *Pseudomonas* and *Bacillus* possess the ability to convert the insoluble phosphate into soluble form by secreting organic acids resulting in improved phosphate availability to the plants. Application of phosphate solubilizers along with vesicular arbuscular mycorrhizae (VAM) improves the phosphorus nutrition of the plants.

In addition, these microorganisms are capable of producing growth hormones such as auxins, cytokinins and gibberellin, vitamins, antibiotics, siderophores *etc.* Due to this property they enhance plant growth and suppress the soil born pathogens. They also improve physico-chemical properties of the soil and sustain soil fertility.

Though almost all the soil have these types of beneficial organisms, their population will most often be at suboptimal level. Hence their activity may not be sufficient to yield significant benefit to the plants. Further, the bioefficacy of all the strains may not be the same. Addition of potential strains of these organisms to the soil becomes essential in this context. For this, efficient strains of organisms have to be found out, mass multiplied and incorporate into soil. By the application of biofertilizers, the chemical fertilizers can be reduced considerably in the fertilizer schedule.

Tea is the most popular beverage produced from the shoots of the commercially cultivated tea plants (*Camellia sinensis* (L.) O. Kuntze). Tea is grown in more than 50 countries, mostly in plantation (Plate 1). It prefers a warm humid climate with well distributed rainfall and long sun shine hours. India with around 4,40,000 ha under tea cultivation is the largest producer and consumer in the world. The world annual tea production reaches 2.5 million tones and an average of 2 billion cups of tea is drunk every day (Baby, 2004).

In India, tea is cultivated in the north eastern region particularly Assam and West Bengal, besides in certain pockets in Himachal Pradesh and Uttar Pradesh. Tea gardens of southern India are spread in Western Ghats in the three states Karnataka, Kerala and TamilNadu.

Tea is a foliage crop where the young, immature shoots containing 2 - 3 leaves and a bud are being harvested at regular intervals. Constant uptake of nutrients to produce more crop results in nutrient depletion in soil and hence adequate inputs of nitrogenous, phosphatic and potasic fertilizers are essentially needed to sustain yield. No single sources of nutrient can meet the nutrient requirement for sustainable productivity of tea. Eco-friendly farming practices utilizing the biological sources and reduced usage of chemical fertilizers are needed to prevent the depletion of plant nutrients and to maintain soil fertility to achieve higher productivity.

Exploitation of biofertilizers in plantation crops like tea was not yet been explored. Tea is a non-leguminous crop which hosts colonizing asymbiotic nitrogen fixers and phosphate solubilizing bacteria in its rhizosphere. Though commercial formulations of these biofertilizers are available most of them were not surviving in soil due to their poor acid tolerance nature. In this context the present study was taken up with the following objectives:

- To isolate the nitrogen fixers and phosphate solubilizers from various agroclimatic zones of the tea plantations of southern India
- To screening the efficient strains suited to different agroclimatic zones
- To identify the elite strains of *Azospirillum* and PSB
- To study the biology and ecology of selected strains
- To study the biodiversity among selected strains of *Azospirillum* and PSB
- To mass multiply the effective strains
- To study the nursery and field performance of biofertilizers in tea

SCOPE OF THE PRESENT STUDY

Exploitation of biofertilizers in plantation crop like tea has not yet been explored. Commercial strains of *Azospirillum* and phosphate solubilizing bacteria were not surviving in tea soil because of their poor acid tolerance nature. So, acid tolerance strains of nitrogen fixers and phosphate solubilizers are essential for tea soils.

There is a need to study the biological and ecological aspects of beneficial organisms to understand the better performance under new environment. For that, the biological and ecological studies were undertaken with selected strains of *Azospirillum* and PSB.

Among beneficial microorganism, there exists a genetic variation. To find out genetic diversity, a study was under taken with selected strains using PCR based RAPD technique.

The nature of tea soil varied widely in different agroclimatic zones. So, selection has to be based on the nature of soil of the agroclimatic zone. Soil is a competitive environment. So, the applied microorganisms should be competitive with native microbial population. For this, addition of sufficient biofertilizers is required for getting maximum response.

Strains developed for other crops were not found suitable in our earlier field experiments. With native efficient stains, field experiments were conducted with various objectives such as standardization, optimization of biofertilizer both nursery and field conditions, method of application of biofertilizers in tea *etc.*

The performance of biofertilizers can be assessed only in long term investigations extending over one or more puring cycle. So, the field experiments were conducted in longer term.

With the onset of liberalization, the Indian tea industry is on the threshold of a new global competition and therefore, there is an urgent need to focus on retaining and improving the competitive advantages. This can be achieved only by improving the productivity and quality. In this context, production of organic tea assumes considerable significance.

REVIEW OF LITERATURE

Biofertilizer is a wide term which includes a diverse category of bioinoculants such as nitrogen fixers, phosphate solubilizers, phosphate mobilizers and plant growth promoting rhizobacteria (Tilak and Singh, 1994). The concept of biofertilizers was developed with the discovery of nitrogen fixing *Azospirillum* by Dobereiner and Day (1976) and phosphate solubilizers by Pikovskaya (1948). These are organisms which are naturally present in all types of soil. But their population level varies with soil and agroclimatic zones. Further, the population level most often may not be enough to bring out significant contribution to the plant nutrition as their efficiency varies with strains. Application of biofertilizers is an environment friendly means to supplement nutrient to the plants. Important biofertilizers are nitrogen fixers, phosphate solubilizers and phosphate mobilizers (Baby, 2002; Jayaraj *et al.*, 2004).

2.1. Nitrogen Fixers

Nitrogen fixers are organisms which assimilate molecular nitrogen present in the atmosphere to ammoniacal nitrogen. *Azospirillum, Rhizobium, Azotobacter* and *Azolla* are the most important nitrogen fixers. Biological nitrogen fixers offer economical, attractive and ecologically sound means of reducing nitrogenous fertilizers.

The nitrogen fixers are classified into symbiotic and asymbiotic nitrogen fixers. *Rhizobium, Bradyrhizobium, Azorhizobium etc.,* are symbiotic nitrogen fixers, which invade the root hairs and cortical tissues and induce the formation of nodules. *Azospirillum, Azotobacter etc.,* are asymbiotic nitrogen fixers, which are free living and closely associated with the plant root system. Among nitrogen fixers, the genus *Azospirillum* received more attention owing to their diazotrophic association with the roots of crop plants and their potential as bioinoculants for crops with promising results.

Azospirillum is a rhizosphere bacterium colonizing the roots of crop plants making use of root exudates and fixes substantial amount of atmospheric nitrogen. They exert beneficial effects on growth and yield of many economically important crops (Okon and Vanderleyden, 1997). They are extensively used in rice and other cereal crops as biofertilizers (Lippi *et al.,* 2004).

Azospirilla are gram negative, motile, curved rod of variable size, ranging from 0.5 – 1 μm in length, exhibit spirillar movement and polymorphism, containing poly -β-hydroxy butyrate (PHB) granules and fat droplets (Okon *et al.,* 1976; Day and Doberiner, 1976). They have peritrichous or polar flagella and are strict microaerobes (Elmerich, 1984; Lamm and Neyra, 1981). They produce thick - walled, cyst like ovoid structures under prolonged incubation and cells are able to aggregate under certain environmental conditions, leading to the formation of bacterial flocs (Burdman *et al.*, 1999).

There are seven species of *Azospirillum*. They are *A. brasilense, A. lipoferum, A. amazonense, A. halopraeferans, A. irakense*, *A. dobereinerae* and *A. largomobile.* The last two species were included in the list very recently. *Azospirillum dobereinerae* was isolated from the roots of graminaceous plant Miscanthus (Eckert *et al.,* 2001), while *A. largomobile* was technically transferred from another genus *Congiomeromonas* based on certain phylogenetic relationships (Dekhil *et al.*, 1997).

Azospirillum population ranged from 10_4 to 10_6 cells per gram of dry soil or root (Rennie, 1980; Magalhaes *et al.,* 1981). Rao and Venkateswarlu (1985) reported that the most probable number of *Azospirillum* varied in the root zone of pearl millet with the maximum in the rhizosphere compared to rhizoplane and inside roots. Further, its population was found to be maximum in laterite soil and the minimum in extremely acid sulphate saline kari soil (Charyulu and Rao, 1980).

Colonization of *Azospirillum* in the roots was known to be non-homogenous. The bacterial cells were observed the entire root system of many plant species, however it preferred root tips and zone of elongation (Levanony *et al.,* 1989; Bashan *et al.,* 1986). The colonization of root tips is advantageous for *Azospirillum* cells because when the roots penetrate deeper into the soil layer, the oxygen supply was lower and competition with the aerobic bacterial population of the rhizosphere was therefore reduced, however analysis of *Azospirillum* sites along the roots revealed that they are found particularly on young roots and much less frequently on the older parts of the roots (Bashan and Levanony, 1987; Cohen *et al.,* 2004).

Attachment of *A. brasilense* to wheat (Michiels *et al*., 1990) and maize roots (Jofre *et al*., 1996; Bashan, 1986b) taken place in two steps. First, bacteria adsorbed rapidly on the root surface. In this step, a protein cell surface component was used for adsorption. In the second step, the adsorption was mediated by surface polysaccharides (exopolysaccharides) or lipopolysaccharides produced by plants (Schloter *et al*., 1984).

Some *Azospirillum* strains penetrate the roots of their host and become endophytes. In such cases, they establish in the intercellular spaces between the epidermis and the cortex (Okon *et al.,* 1977b; Dobereiner, 1983b; Whallon *et al.*, 1985) or even in the vascular system (Patriquin *et al.,* 1983; Baldani *et al.,* 1983). Bashan and Levanony (1988) reported the colonization of *A. brasilense* in the endodermis of wheat roots.

2.2. Phosphate Solubilizers

Phosphorus is one of the major plant nutrients required in optimum amount for plant growth. Some heterotrophic bacteria and fungi are known to have the ability to solubilize inorganic phosphate from insoluble sources and making available to plants. These microorganisms are known as phosphate solubilizers. The most efficient phosphate solubilizing bacteria include *Bacillus* and *Pseudomonas* and that of fungi include species of *Aspergillus* and *Penicillium* (Baby, 2002).

Pikovskaya (1948) was made a pioneering attempt in isolating an organism capable of actively solubilizing tricalcium phosphate and coined the name "Baterium P". Later he had formulated a medium having glucose as carbon source and ammonium sulphate as nitrogen source with yeast extract and tricalcium phosphate for the isolation of phosphate solubilizing bacteria. Enrichment technique and special media for the isolation of acid producing and phosphate dissolving microorganisms from soils and rhizosphere were designed by Sperber (1958b) and Katznelson and Bose (1959). Sackett *et al.* (1908) used the agar plate technique and provided conclusive evidence to show that soil bacteria dissolved dicalcium phosphate, tricalcium phosphate, bone meal and rock phosphate.

Phosphate solubilizing bacteria were found in all soils but their number varies with soil, climate as well as crop history (Gupta *et al*., 1986). Soil samples collected from sugarcane growing belt of north Bihar indicated that the population level of PSB ranged from 27 - 112 x 10^3 per gram soil. This large variation in their distribution in different soils might be due to the differences in organic carbon content (Yadav and Singh, 1991). Mahmoud *et al*. (1973) made a comparative study of the population of PSM in the rhizosphere of broad bean and wheat. Broad bean had more population of phosphate solubilizers than the wheat plants.

Bacteria, fungi and actinomycetes are active in solubilizing insoluble inorganic phosphate with high efficiency (Kapoor *et al*., 1989; Narsian *et al*., 1994; Narsian and Patel, 1995). Frietas and Germida (1990) isolated the phosphate solubilizing microorganisms such as *Pseudomonas* a*eruginosa*, *P. cepacia, P. fluorescens* and *P. putida* from the rhizosphere of wheat and *Bacillus licheniformis, B. mycoides, B. megaterium* from the rhizosphere of paddy (Watanabe and Hayano, 1993).

2.3. Biology and Ecology of *Azospirillum* and PSB

Soil is a habitat for varied group of microorganisms and their activity largely determines the physicochemical properties of the soil and growth of the plants. The biological activities in soil are markedly influenced by two or three member association and such syntrophic associations are of ecological importance with implied agricultural significance. *Azospirillum* and PSB are diazotrophic bacteria associated with almost all types of crop plants. These organisms supply N and P to the plants by fixing atmospheric nitrogen and P solubilization respectively. They are also able to increase plant growth by producing growth promoting substances and protect the plants from pathogens by suppressing their growth.

2.3.1. Biology of *Azospirillum*

Azospirillum grown in N-free medium behaves as microaerophilic and fixes nitrogen and when supplemented with nitrogen it grows as an aerobe (Day and Dobereiner, 1976). In culture tubes of semisolid medium with a suitable carbon and energy source, *Azospirillum* develops a growth pellicle just below the surface and fix the nitrogen only under microaerophilic condition, because its nitrogenase is poorly protected from oxygen (Okon *et al.,* 1977a; Nelson and Knowles, 1978).

Kundu (1992) reported that the nitrogen fixation was highest in semisolid followed by solid and liquid medium. Yeast extract in medium increased the acetylene reduction than vitamins and the higher concentration showed a decline due to availability of fixed nitrogen in the medium. Isolates of *A. lipoferum* exhibited a higher nitrogenase activity (67.6 n mole C_2H_4 mg protein^{-1}h^{-1}) compared to *A. brasilense* (39.2 n mole C_2H_4 mg protein^{-1}h^{-1}) in nitrogen free medium (Han and New, 1998).

Azospirillum prefers acidic pH for their growth and activity. The optimum pH for the growth of *A. amazonense, A. lipoferum* and *A. brasilense* strains isolated from a variety of habitats found to be 5.7 - 6.5, 5.7 - 6.8 and 6.0 - 7.3 respectively (Baldani *et al.,* 1986b). *A. brasilense* strain sp 7 was reported to fix nitrogen at the extremes of pH 4.5 and 9.2 (Das and Mishra, 1983).

Soil pH was one of the important factors deciding the nitrogenase activity of *Azospirillum* (Dobereiner *et al.*, 1976; Boddey and Dobereiner, 1988). A decrease in the nitrogenase activity was recorded when the pH was raised to 7.8. An optimum pH of 7.1 to 7.4 was recorded by Okon *et al.* (l977a) for the growth and nitrogenase activity of *Azospirillum* isolates, while Das and Mishra (1983) reported it to be pH as 7.0 to 7.5. Charyulu and Rao (1980) found that isolates originated from soil having low pH (< 4.0) had lower nitrogen fixation than those from soils of higher pH up to 6.6. Karthikeyan (1994) isolated acid tolerant *Azospirillum* that could fix nitrogen at pH levels of 4.5 to 6.0.

The optimum temperature for growth of *Azospirillum* was found to be between 32 and 40°C (Day, 1977), while for nitrogen fixation it was below 24°C (Day and Dobereiner, 1976; Okon *et al.*, 1980) and 31°C (Neyra and Dobereiner, 1977).

Azospirillum brasilense, *A. lipoferum* and *A. halopraeferens* showed optimum acetylene reduction activity at 25, 30 and 40°C respectively. In *A. brasilense* and *A. lipoferum*, temperature above 35°C inhibited both *nif* gene expression and acetylene reduction activity (Tripathi and Klingmuller, 1992; Alenkina *et al.*, 2007). A little fluctuation of temperature with in this range would not alter nitrogen fixation (Albrecht *et al.*, 1977). Purushothaman and Vijila (1989) isolated a few azospirilla strains that were capable of fixing nitrogen 50°C.

A wide variety of microorganisms were known to produce poly β-hydroxy butyrate (Williams and Peoples, 1996). Poly -β- hydroxy butyrate (PHB) is a common food reserve in bacteria and it was believed that PHB accumulation give resistance to temperature (Tal and Okon, 1985). *Azospirillum brasilense* accumulated PHB up to 70 per cent of its dry weight (Okon and Itzigsohn, 1992). Purushothaman and Vijila (1989) observed a phenomenal accumulation of PHB when in the *Azospirillum* cells grown at 50ºC. *Azospirillum* produced high level of poly -β- hydroxy butyrate under suboptimal growth conditions. Utilization of PHB under stress condition is a mechanism that favours their establishment (Kadouri *et al.,* 2002).

Exopolysaccharide production is an important trait of bacteria because it protects the cells against desiccation, phagocytosis and phage attack and helps in nitrogen fixation by preventing high oxygen tension (Jarman *et al*., 1978). Exopolysaccharides help the bacteria to firmly attach on the root surface (Croes *et al.,* 1993; Michiels *et al.*, 1991). *Azospirillum* spp. produced significant quantity of exocellular and capsular polysaccharides (Ketalogianni and Aggelis, 2002). Production of EPS was higher in *Azospirillum* isolate than *Pseudomonas* isolate (Tank and Saraf, 2003). They are also able to produce siderophores.

Azospirillum brasilense failed to grow on glucose, sucrose or α-keto glutarate (Tarrand *et al.*, 1978; Krieg and Dobereiner, 1984), a property normally found only in *A. amazonense* (Goebel and Krieg, 1984; Loh *et al.*, 1984; Baldani *et al.*, 1986a). Day and Dobereiner (1976) reported that sugar was poor substrate for *A. brasilense* but better source for *A. lipoferum*. The growth response of *Azospirillum* strains indicated that D-fructose, D-mannitol, sorbitol, sucrose, tyrosine and tryptophan were poor carbon sources, while α-keto glutarate, L-alanine, L-glutamate, lactate, pyruvate and succinate were good carbon sources (Rai and Gaur, 1982; Del Gallo *et al.*, 1984).

Azospirillum spp. differed in their utilization of amino acids. *A. lipoferum* and A. *brasilense* readily utilized many amino acids as the sole source of carbon and nitrogen. Glutamate was a good nitrogen source for *A. lipoferum* and *A. amazonense*, but a poor source for *A. brasilense* and *A. halopraeferens*. *Azospirillum amazonense* and *A. lipoferum* utilized amino acids such as glutamate, aspartate, serine, and histidine as sole carbon source, but *A. brasilense* and *A. halopraeferens* were unable to utilize these amino acids but readily utilized alanine (Hartmann, 1988).

The acetylene reduction was enhanced with various nitrogen sources. Inorganic nitrogen sources caused significant reduction in nitrogenase activity, while organic sources such as amino acids and protein either stimulated or did not significantly inhibit the activity, however urea was completely inhibitory (Rao and Venkateswarlu, 1982). Biological nitrogen fixation in a plant soil system was strictly dependent on the soil oxygen tension and the concentration of mineral N (Alexander *et al.*, 1987).

Agrochemicals especially pesticides and herbicides have an influence on the growth of *Azospirillum* (Gadkari, 1987). Incorporation of insecticides in the growth medium caused either cell disruption or formation of cyst-like bacteria (Buff *et al*., 1992). The herbicide 2, 4 -dichlorophenoxyacetic acid (2, 4-D), at concentrations used on crops, directly altered the attachment of *Azospirillum brasilense* Cd to maize roots (Jofre *et al*., 1996). At very low concentration of 1 mM 2, 4-D inhibited *Azospirillum* cell growth (Bashan and Holguin, 1997).

Fungicides like sulphur, Dithane M-45, Captan, Bavistin and Benlate at normal doses of application were found stimulatory but Vitavax, Agrosan GN were inhibitory (Zambre and Konde, 1985). Pesticides generally reduced the population level and varied with the types of pesticides (Bezbaruah, 1999).

Dobereiner and Baldani (1979) found diversity among *Azospirillum* strains with respect to their resistance to various antibiotics. *Azospirillum amazonense* strains were resistant to penicillin but relatively tolerant to chloramphenicol and erythromycin (Magalhaes *et al.*, 1983). Govindarajan and Prushothaman (1984) reported that out of 80 isolates of *Azospirillum* only one isolate was resistant to 50 µg ml^{-1} of streptomycin. Fifteen per cent of isolates exhibited resistance up to 20 µg ml^{-1} of streptomycin and 7 per cent tolerated only up to 1.0 µg ml^{-1}. Merline Sheela (1991) studied the resistance of *Azospirillum* isolates to various antibiotics and found that their tolerance level was streptomycin (150 µg/ml), rifampicin (3 µg/ml), .chloramphenicol (150 µg/ml), tetracycline (5 µg/ml), kanamycin (20 µg/ml), ampicillin (100 µg/ml), erythromycin (20 µg/ml) and neomycin (10 µg/ml).

2.3.2. Biology of PSB

PSB were able to produce halo zone of P solubilization around the colonies in solid medium. This criterion used as a method to isolate phosphate solubilizing microorganisms (Peix *et al.*, 2003).

The degree of solubilization was influenced by pH, EC, O_2 and CO_2 concentration and by the presence of organic material (Bhattacharyya and Jain, 2000). The factors like nutrition, aeration and temperature greatly influenced the phosphate solubilization (Illmer and Schinner, 1992). The solubilization of different types of insoluble phosphate was varied with the type of microorganisms, nature of phosphate, media conditions and available carbon sources (Yadav and Dadarwal, 1997). The reduction of pH in the culture medium accounted for best solubilization of various insoluble P sources such as MnO_2, Fe_2O_3, CuO, granular metallic zinc and rock phosphate (Altomare *et al.*, 1999). A fall in pH accompanied phosphate solubilization due to the production of organic acids, but no correlation could be established between acidic pH and quantity of P_2O_5 liberated (Dave and Patel, 1999; Whitelaw *et al.*, 1999; Bar-Yosef *et al.,* 1999; Rashid *et al.,* 2004). Maximum solubilization of phosphate by PSB was found to be at near neutral pH, while for fungi it was at 4.0 to 5.0 (Whitelaw, 2000; Pradhan and Sukla, 2005).

Acid and neutral pH influenced the production of acid phosphatase, whereas alkaline phosphatase favoured alkaline pH (Dick and Tabatabai, 1984). The pH change in culture medium was due to production of organic acids and this support the solubilization of insoluble phosphate. The pH change was differed based on the type of organic acids produced. A correlation was detected between media pH and titratable acidity (Reyes *et al.*, 1999; Ivanova *et al.,* 2006). Son *et al.* (2003) reported that the initial pH of 7.5 was reduced to 2.6 due to growth of PSB in culture medium.

Phosphate solubilization was greatly influenced by the temperature of culture medium. A range of 30 - 35ºC was more favorable for rock phosphate dissolution by PSB in the alkaline soils of tropics. Narula *et al.* (1995) isolated PSB from the soil of oil seed crops which were able to solubilize tricalcium phosphate and rock phosphate at 37ºC and 42ºC respectively. Wani *et al.* (1979) reported that the selected PSB cultures in liquid medium solubilized tricalcium phosphate and rock phosphate at a temperature range of 30 - 35ºC. *Swaminathania salitolerances* isolated from wild rice (Loganathan and Nair, 2004) solubilized phosphate at 30ºC, while PSB isolated from chickpea solubilized P at 45ºC (Surange *et al.*, 1997; Whitelaw *et al.,* 1999; Gadagi and Sa, 2002). Solubilization was negligible at lower temperatures and there was a delay in the expression of phosphatase activity at lower and higher temperatures (De Sousza *et al.,* 2000; Gaind and Gaur, 1999).

PSB were able to produce exopolysaccharides in the culture media. Exopolysaccharide production was an important trait of beneficial microorganisms because it protects the cells against desiccation, phagocytosis and phage attack (Jarman *et al.*, 1978). Incubation time was greatly affected the production of exopolysaccharides by PSB in the culture medium. Maximum production was after 120 h of incubation. The strains *Bacillus* TB1, TB2 and TB3 were produced higher amount of exopolysaccharides compared to strains of *Pseudomonas* TP1, TP2 and TP3 (Tank and Saraf, 2003).

Phosphate solubilizing microorganisms are known to produce iron chelating substance, namely siderophores, which has great impact on plant growth promotion, iron nutrition and suppression of phytopathogens (Chincholkar *et al*., 2000; Leong, 1986). They are low molecular weight iron binding compounds synthesized in large amount and excreted into culture medium by microorganisms under iron deficient conditions. These are common products of aerobic and facultative anaerobic bacteria and fungi.

Fluorescent *Pseudomonas* produced fluorescent yellow green, water-soluble siderophores, pyoverdine (Meyer and Abdallah, 1978; Kleeberger *et al.*, 1983). Dileepkumar and Bezbaruah (1996) reported the presence of hydroxyamate siderophores in the crude extract of *Psuedomonas* strains isolated from rhizosphere of tea. Hydroxamate-type siderophores were stable in soil over a wide range of pH (Neilands, 1981; Weibe, 2002).

Azospirilla also produces siderophores, but in lesser quantity compared to pseudomonads. *Azospirillum* spp. produced salicylate type of siderophores ranged from 1.28 - 3.20 mg/l (Sridhar and Balasubramanian, 1996). Saxena *et al*. (1986) reported that under iron limiting conditions *A. lipoferum* D2 excreted phenolate type of siderophores, *A. lipoferum* M produced catechol–type siderophores (Shah *et al.*, 1992). Tapia-Hernandez *et al.* (1990) reported that 27 strains of *Azospirillum* produced bacteriocins type siderophores.

Siderophore production was influenced by a great variety of factors *viz*., concentration of iron (Klopper *et al*., 1980), nature and concentration of carbon and nitrogen sources, level of phosphates (Barbhaiya and Rao, 1985). Presence of trace element such as magnesium (Georgia and Poe, 1931), zinc (Chakrabarty and Roy, 1964) and molybdenum (Lenhoff *et al*., 1956) also enhances siderophore production. Maximum synthesis occurred when culture medium contained malic, succinic and gluconic acids served as the carbon source, whereas citric acid had the inhibitory effect (Suslow, 1982).

The nature of carbon sources greatly affected the phosphate solubilization under *in vitro* and *in situ* conditions and was more active in presence of hexoses and pentoses or disaccharides. Effective phosphate solubilizers were selected on the basis of their ability to solubilize insoluble tricalcium phosphate and rock phosphates in liquid medium having a suitable easily metabolizable carbon substrate. Production of organic acid mainly mono-di and tri-carboxylic acids and gluconic acid were often determined in the liquid broth and siderophore production ability (Dadarwal *et al*., 1997; Ponmurugan and Gopi, 2006).

Pseudomonas fluorescens utilized variety of carbon compounds as energy source. Among carbon sources, glucose was found as the best followed by galactose for *P. fluorescens* (Dave and Patel, 2003), where as glucose followed by sucrose and galactose for *P. striata* (Gaur and Sachar, 1980). Gyaneshwar *et al*. (1998) found that *Enterobacter asburiae* solubilized phosphate when grown in presence of glucose as carbon source and ammonium/nitrate as nitrogen source. Release of phosphorus was enhanced with increasing concentration of glucose and this was attributed to greater availability of the energy source for the growth of organisms and acid production. Solubilization was maximum at 2 per cent concentration compared to lower concentration (Yadav and Singh. 1991). Monosaccharides were superior to disaccharides and polysaccharides for the solubilization of rock phosphate by *Pseudomonas fluorescens.* Among monosaccharides, hexoses were better than pentoses (Dave and Patel, 2003).

PSM produced significant quantity of organic acids as metabolic by-products depending on various carbon sources (Bhattacharyya and Jain, 2000). Among three sugar alcohols, glycerol was found best for phosphorus liberation from both tricalcium phosphate and rock phosphate (Dave and Patel, 2003).

Inorganic nitrogenous compounds supported phosphate solubilization more efficiently compared to organic compounds (Vora and Shelat, 1998; Dave and Patel, 2003). Gaur and Sachar (1980) observed a remarkable increase in available phosphorus in soil amended with ammonium sulphate and PSM.

2.4. Ecology of *Azospirillum* and PSB

Azospirilla were widely distributed in various geographical regions and tropical fertile soils (De Silva and Dobereiner, 1978; Dobereiner and Pedrosa, 1987; Bashan and Levanony, 1990). They were isolated from the rhizosphere of a wide variety of plants including grasses and cereals from tropical and temperate regions, cold climatic zones from desert plants, water- flooded rice paddies and salt-affected soils (Haahtela *et al.,* 1981; Reinhold *et al.,* 1987; Linderberg and Granhall, 1984). However, the rhizospheres of only some tropical vegetation were quantitatively important for azospirilla. In temperate and cold habitats they were out numbered by other rhizosphere microorganisms (Michiels *et al.,* 1989).

Survey on natural occurrence of *Azospirillum* suggested that they were common rhizosphere residents in the tropics, but much less prevalent in temperate soils (Dobereiner, 1983a; Pedrazal *et al*., 2007). On the contrary, Vlassak (1981) claimed that the genus is widely distributed in temperate soils. Lindberg and Granhall (1984) were of opinion that the genus *Azospirillum* was not a major constituent of the temperate soils, where the genera

Pseudomonas and *Enterobacter* were the dominant microflora Therefore, large screening programmes were undertaken to isolate indigenous plant growth promoting rhizobacteria having strong root colonization (Michiels *et al.*, 1989).

There were several reports on the occurrence and distribution of nitrogen fixing bacteria in the rhizosphere of plants (Diem and Dommergues, 1979), but these studies confined only to grasses and other annual crops and very little attention had been paid to perennial and orchid plants (Subba Rao, 1983; De Silva and Dobereiner, 1978). Charyulu and Rao (1980) reported the occurrence of *Azospirillum* species with appreciable nitrogen fixing ability in an acid sulfate soil with an extremely low pH of 3.2. Alkaline and sodic soils, saline soils, soils of deserts and sand dunes either did not support *Azospirillum* or harboured only a low population (Michiels *et al*., 1989). Plantation crops like arecanut, cashew, cocoa, rubber, cardamom and sapota grown in acid soils colonized *Azospirillum* in their root system (Govindan and Purushothaman, 1985).

Phosphate solubilizing bacteria were also distributed in all types of soils. Their population was influenced by soil type and cultivation practices, humus content, N, P and K level (Kobas, 1962; Calvaruso *et al*., 2007). There was no correlation with vegetation type and quantity of phosphate solubilizing microorganisms. Black soil had significantly higher proportion of phosphate solubilizing microorganism than dark brown or brown soil (Kucey, 1983).

The establishment and performance of PSB were affected severely under abiotic stress prevalent in degraded ecosystem *viz.* alkaline soils with a tendency to fix phosphorus

(Surange *et al.,* 1997; Fankem *et al.,* 2006). Phosphate solubilizing bacteria like *Pseudomonas aeruginosa, P. cepacia, P. fluorescens, P. putida,* from the rhizosphere of wheat (Frietas and Germida, 1990; Gomathy *et al*., 2007), *Bacillus megaterium, B. brevis, B.cirulans B. subtilis* from the rhizosphere of oats (Gaind and Gaur, 1999), *Bacillus licheniformis*, *B. mycoides*, *and B. megaterium* from the rhizosphere of paddy (Watanabe and Hayana, 1993). Johri *et al.* (1999) found greater number of phosphate solubilizing microorganisms in rhizoplane and rhizosphere than root free area of *Prosopis juliflora.* It was also reported that the organisms obtained from the rhizosphere of crop plants solubilized more of tricalcium phosphate compared to those obtained from non-rhizosphere soils (Craven and Hayasaka, 1982).

2.5. Mechanism of nitrogen fixation

The genes associated with nitrogen fixation are designated as *nif.* Studies on the genetics of nitrogen fixation were carried out in *Klebsiella pneumoniae* in which the organization of the *nif* genes was well understood. The *nif* genes were clustered into a single regulon containing 23280 bp. This entire region had been sequenced. It consists of 20 genes organized most probably into seven operons. The genes of *nifH, nifD* and *nifK* encoded the structural proteins of Fe and MoFe proteins of nitrogenase. These genes are highly conserved among diazotrophs, emphasizing the similar nature of the enzymes in various nitrogen fixing organisms. *nif* genes involved in the synthesis and regulation of nitrogenase (Arnold *et al.,* 1998). The genes of *nifT, nifX* and *nifZ* have no major role in nitrogen fixation. It has recently been proposed that the *nifW* gene product may be involved in stabilizing the MoFe protein, possibly against oxygen damage (Dixon *et al.,* 1997).

The biological nitrogen fixation requires a complex enzyme system since the reaction is highly endergonic. The protons and electrons required for this process are generated in metabolic reactions and catalyzed by an enzyme nitrogenase. It is found only in prokaryotic microorganisms and so eukaryotes can benefit from nitrogen fixation only if they interact with nitrogen fixing prokaryotes to obtain the fixed nitrogen after their death and decomposition.

Nitrogenase enzyme is highly essential for reducing nitrogen to ammonia and is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase). The individual proteins do not catalyse any reaction. The Fe and Mo-Fe protein of nitrogenase have been sequenced and characterized from a variety of nitrogen fixing bacteria (Burgess and Lowe, 1996).

Fixation of atmospheric N by *Azospirillum* was evaluated mainly by acetylene reduction assay and this method was useful for quantitative evaluation of N fixation by *Azospirillum* and their screening (Van Berkum and Bohlool, 1980).

2.6. Mechanism of phosphate solubilization

The main mechanism in solubilizing insoluble phosphate by soil microbes is on their ability to secrete organic acids. The organic acids bring down soil pH resulting in the dissolution of immobile forms of phosphate (Hegde and Dwivedi, 1994). Phosphate solubilizing microorganisms were found to produce mono carboxylic acids (acetic, formic), monocarboxylic hydroxy acids (lactic, gluconic) dicarboxylic acids (oxalic, succinic), dicarboxylic hydroxy acids (malic, maleic) and tricarboxylic hydroxy acids (citric) in liquid medium from simple carbohydrates (Sperber, 1958a). A fall in pH accompanied phosphate solubilization due to the production of organic acids, but no correlation could be established between acidic pH and quantity of P_2O_5 liberated (Dave and Patel, 1999).

The type of organic acids produced and their quantity differ with different microorganism. Tri and di-carboxylic acids are more effective as compared to mono basic and aromatic acids. Aliphatic acids are also found to be effective in P solubilization than phenolic acids. Citric acid and fumaric acids had highest P solubilization ability (Kapoor *et al.*, 1989).

Acidic and neutral pH influenced the production of acid phosphatase, whereas the alkaline phosphatase was produced in alkaline pH (Dick and Tabatabai, 1984; Chunlei *et al.*, 2007). Under *in vitro* conditions, pH 6.0 favoured acid phosphatase secretion (Boquet *et al.*, 1987), whereas it was pH 7.3 for alkaline phosphatase (Magnouloux and Portalier, 1988).

2.7. Plant growth promotion

2.7.1. Plant growth-promoting rhizobacteria

The rhizosphere is one of the largely unexplored frontiers in plant-microbes interaction. The rhizosphere microbial population and communities influenced on plant nutrition, plant diseases and root architecture (Barea *et al.,* 1976; O’Connel *et al.,* 1996; Patten and Glick, 2002). In recent years, more attention is being given for searching early root colonizers which directly or indirectly benefit plant growth. Beneficial root associating soil bacteria are usually referred to as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper *et al.,* 1989; Piao *et al.,* 1992; Ona *et al.,* 2005; Somers *et al.,* 2005). Among the root associated bacteria pseudomonads are the early root colonizers, which contribute considerably to plant production and protection. Plant growth promoting rhizobacteria belong to several genera *viz., Azospirillum, Azotobacter, Acetobacter, Bacillus, Bradyrhizobium, Erwinia, Pseudomonas, Rhizobium* and *Serratia.*

The beneficial effect of PGPR is attributed to increase the nitrogen input from biological nitrogen fixation, higher phosphate solubilization, production of plant growth promoting hormones like auxins, gibberellins and cytokinins and reduction of plant diseases and nematode infection. Beneficial effects reflect as direct plant growth promotion and/or inducing host resistance. Specific PGPR strain brings about Induced Systemic Resistance (ISR) against multiple pathogens attacking the same host. Broad spectrum of disease control using PGPR strains is an effective and economical way for plant protection. Moreover, certain PGPR strain mixture showed the synergistic action in growth promotion as well as plant protection.

Phytohormones or plant growth hormones in the rhizosphere are originated from plants through root exudates and also by microbial synthesis *in situ*, of which microbial production is considered as the primary source. The phytohormones induce plant growth as well as dry matter production. Inoculation with *Azospirillum* resulted in better growth and higher dry matter production in maize and was mainly attributed to nitrogen fixation and the production of plant growth regulators (Arshad and Frankenberger, 1991). The major hormone produced was indole -3- acetic acid (IAA) (Fallik *et al.,* 1989). Other hormones detected at much lower, but biologically significant level were indole -3- lactic acid (Tien *et al.,* 1979; Xie *et al.,* 2005), indole -3- methanol (Crozier *et al.,* 1988), unidentified indole compounds (Hartmann *et al.,* 1983), abscisic acid (ABA) (Kolb and Martin, 1985), cytokinins (Horemans *et al.,* 1986), indole -3- butyric acid (IBA) (Fallik *et al.,* 1994) and several gibberellins (Bottini *et al.,* 1989).

Among the phytohormones, the level of gibberellin like substances produced by bacteria in association with roots was low. *A. brasilense* was able to produce gibberellins and cytokinin like substances in the liquid culture (Vlassak and Reynders, 1978). The production of gibberellin was higher in culture medium after two days of inoculation while cytokinins showed an increase after ten days (Cacciari *et al.,* 1989).

Frietas and Germida (1990) showed that the inoculation of *P. aeruginosa, P. cepacia, P. Putida* and *P. fluorescens* strains on winter wheat increased the plant height, root and shoot mass and number of tillers in growth chamber. The available P_2O_5 content of the soil, nodulation, root and shoot biomass, straw and grain yield were increased due to PSM inoculation compared to the uninoculated controls (Gaur *et al.,* 1980; Gaind and Gaur, 1999). Field study with *Bacillus megaterium* and *B. circulans* in rice and wheat significantly increased the yield which was equivalent to the yield obtained with up to 50 kg P_2O_5 /ha (Hedge and Dwivei, 1994). Inoculation of phosphate solubilizing microorganism alone or in combination with phosphatic fertilizers increased the available phosphorus in soil and total P uptake in rice. The beneficial effect was superior to the effect obtained with super phosphate

(Banik, 1982; Souchie *et al.*, 2006). Dubey *et al*. (1997) observed that rock phosphate at 30 kg P_2O_5/ha plus *Pseudomonas striata* was equally effective to single super phosphate at 60 kg P_2O_5/ha in improving the nodulation in groundnut.

2.8. Biodiversity

A number of methods for genotypic typing are available. Earlier approaches, evolved in 1970s were based on the analysis of restriction fragment length polymorphisms (RFLP) by restriction endonuclease digestion of plasmid DNA. Efforts using restriction endonuclease digestion and/or DNA probing techniques came next. The development of pulsed-field gel electrophoresis (PAGE) in the mid 1980s ushered as the next generation. It is a powerful tool for chromosomal comparison. At the same time, the discovery of PCR led to the development of a plethora of amplification based technique. Most recently, fourth generation approaches involving a direct comparison of chromosomal sequences have started to assess potential inter relationships between individual isolates to the most fundamental level. This approach includes RAPD, AFLP *etc.*

It is essential to understand the bacterial community, structure and diversity in relation to environmental factors and ecosystem functions (Torsvik *et al*., 1996). Rangarajan *et al.* (2001) assessed the diversity of the rhizosphere *Pseudomonas* population associated with three different host plants grown in saline soil in order to study the effect of salinity on rhizobacteria. Using PCR finger printing technique a limited genetic diversity was found between *Azospirillum lipoferum and Herbaspirillum seropedicae* (O’ Callaghan *et al.*, 1994).

RAPD is a PCR-based assay used in genetic analysis with single arbitrary short oligonucletide primers (9 - 10 bp) in an exponential manner. It has got wide acceptance principally due to the easiness of the procedure and the very low amount of DNA required for analysis (Buescher *et al.*, 1994). It is also used to assess intra or inter population genetic variability (Lodhi *et al.*, 1997; Hartskeerl *et al.*, 1989) and to identify molecular markers linked genes of interest (Kirchoff and Hartmann, 1992). In RAPD analysis, the variation within the groups was also quite large and helpful not only in defining sub-groups but also in defining the distance among isolates from standard strains (Suman *et al.*, 2001; Erlich *et al.*, 1991).

The diversity study within a targeted bacterial population helped to screen the genotypes that were best adapted to particular environmental stresses or ecological habitats (Achouak *et al.*, 2000). Diversity within certain species of *Azospirillum* (Fani *et al.*, 1993) and *Bacillus* (Mavingui *et al.*, 1992) has already been established using genomic fingerprinting approaches.

RAPD analysis showed a considerable level of genetic diversity among the strains isolated from different host plants. Soil pH and salinity seems to have an effect on the selection of natural population as revealed by PCR-RAPD (Saleena *et al.*, 2001). It was well known that organic soil had higher diversity than sandy soil due to the higher physico-chemical complexity (Torsvik *et al.*, 1996).

Saleena *et al.* (2001) developed RAPD procedure, in which 10 base pair primers of arbitrary sequence was used to amplify discrete DNA fragment using genomic DNA as template. The level of genetic diversity in nitrogen fixing bacteria in relation to their habitat was obtained by LINE-PCR fingerprinting (Mc Arthur *et al.*, 1988). The dendrogram constructed based on the dissimilarity coefficient showed grouping of the isolates into different clusters based on the existence and occurrence (Loganathan *et al.*, 1999). They have found 60 per cent dissimilarity among *Pseudomonas* associated with pearl millet, cotton and paddy.

2.9. Mass Multiplication

Various delivery systems such as agar slope based culture, liquid paraffin covered agar cultures, liquid culture in polythene bags and carrier based cultures have been used for the supply of microbial inoculants (Bashan and Holguin, 1997). For large scale production and commercial use in agricultural farms, the carrier based cultures packed in polythene bags with proper sealing, is the common accepted practice in almost all countries. This method has several advantages over others except that the system requires bulk quantities of the product and short shelf life of 3 - 6 months. Longer storage allows proliferation of contaminants and decrease in viability of added microorganisms (Dadarwal *et al*., 1997).

Testing of carrier materials suitable for large scale use is an essential pre-requisite for the successful use of microbial inoculants. A number of carrier materials such as lignite, charcoal, pressmud, agro industrial waste, compost were used for mass multiplication of beneficial microorganisms. The carrier material used for inoculant preparation should have the characteristics like high organic matter content high water holding capacity and neutral pH (Date, 1968). It should also be non polluting, biodegradable, non toxic and cost effective which can maintain high viable count for a longer period (Tilak and Singh, 1994).

The most appropriate carrier material was peat, which was commonly used in USA, Australia and other European countries. In India, its use remains restricted due to limited availability. Lignite and wood charcoal were considered as better alternatives to peat. Lignite had acidic pH and required around 10 per cent (W/W) addition of calcium carbonate to bring pH near neutral. Wood charcoal on the other hand, had pH around 7.5 - 8.0 with water holding capacity equivalent to peat and therefore serves as better carrier. The lignite and wood charcoal were the most commonly used carrier material in India (Dadarwal *et al*., 1997). Lakshmi *et al.* (1977) reported that the organic carbon, nitrogen, and phosphorus content in exfoliated vermiculite were negligible compared to lignite. The poor nutrient content of vermiculite has to be increased by nutrient amendments to support the growth of bacteria. Graham-Weiss *et al*. (1987) reported that when supplemented with nutrient source, sterile finely ground vermiculite can be used as a carrier material. The survival of *Azospirillum* and phosphate solubilizing bacteria was better in press mud compared to peat, lignite, and composted pressmud, but in the field the survival of *Azospirillum* was better in composted pressmud (Rajannan *et al*., 1996). Muthukumarasamy *et al*. (1996) suggested that the vermicompost could be used as an alternative carrier material to lignite.

Combinations of organic materials were tested for their potential by various research institutes and commercial firms. Tilak *et al*. (1979) found that soil plus farmyard manure (1:1 ratio) had higher *Azospirillum* count followed by soil plus FYM plus vermiculite (5:3:2).

Kundu and Gaur (1981) suggested that soil could serve as a carrier for spore formers like *B. polymyxa*. Tilak and Subba Rao (1978) established that lignite, pressmud and farmyard manure after suitable amendment with charcoal or coconut shell powder could substitute peat under Indian condition.

The carrier-based inoculants of the diazotrophs and PSM are normally applied with seeds, seedling tubers, rhizome or cuttings. Direct application of carrier based formulation could also be done but these methods require larger quantities of inoculants and particularly difficult to follow by average farmer (Dadarwal *et al*., 1997). Soil and farmyard manure, mixed 1:1 and sterilized for 3 h daily on three consecutive days was a suitable carrier for *Azospirillum* (Tilak *et al.,* 1979).

For the large-scale production of *Azospirillum,* Okon's medium having malate as carbon source and yeast extract as vitamin source and for PSM, Pikovskaya's medium having glucose as carbon source, ammonium sulphate as nitrogen source with yeast extract and tricalcium phosphate were used (Pikovskya, 1948). The media pH was kept around 7.0 with incubation temperature 30°C for production of PSB in large scale (Dadarwal *et al*., 1997).

Quality of the bioinoculants is one of the most important factors deciding the field performance of bioinoculants (Baby, 2004). The quality of culture broth depends on the viable cell count. A viable cell count ranging from 10^8 to 10^9 per gram of carrier based formulation was often desired to have a satisfactory microbial load (Dadarwal *et. al*., 1997).

2.10. Organic farming

The major objective of organic farming is to reduce the chemical inputs and sustain crop production. One way of achieving this objective is through integrated nutrient management rather than sole reliance on chemical fertilizers. No single source of nutrient can meet the nutrient needs of a crop for sustainable productivity. In an ideal system, chemical fertilizers, organic manures and biofertilizers should be given as complementary to each other in a balanced way (Baby, 2002).

Organic farming is a productive system, which reduces or avoids entirely the use of chemical fertilizers and pesticides, growth regulators and other agricultural chemicals. The system relies on crop rotation, organic manure and biofertilizers for nutrient supply, biopesticides and biocontrol for pest and disease control and innovative crop husbandry practices for maintaining soil productivity. Organic farming is best suited for perennial plantation crop like tea, which provide proper shade and cover to the soil and keep the soil microbial activity constant. To keep the soil microbially active, there should be enough organic matter and hence in an organic farming system there is a need for maintaining content by periodical supply.

MATERIALS AND METHODS

3.1. Chemicals and glassware

Analytic grade (AR) chemicals (E.Merk and Himedia) and Schott Duran and Borosil glassware were used throughout the study.

3.2. Cleaning of glassware

The glassware were first cleaned with detergent and then immersed in the cleaning solution over night and washed thoroughly in tap water, rinsed with distilled water and dried in hot air oven.

Cleaning solution

Potassium dichromate	- 80.0 g
Conc. Sulphuric acid	- 400 ml
Distilled water	- 300 ml

3.3. Sterilization

Media and glassware except petri plates were sterilized in an autoclave at 15psi. for 22 min. Petri plates were sterilized at 150ºC in hot air oven, over night.

3.4. Media

3.4.1. Nitrogen free semisolid medium (Dobereiner *et al.*, 1976)

Malic acid	-	5.0 g
Di-potassium hydrogen phosphate	-	0.5 g
Magnesium sulphate	-	0.2 g
Sodium chloride	-	0.1 g
Calcium chloride	-	0.1 g
Potassium hydroxide	-	4.0 g
Yeast extract	-	0.05 g
Bromothymol blue	-	2.0 ml (1% alcoholic solution)
Distilled water	-	1000 ml
Agar*	-	1.75 g (semi solid medium)
pH	-	6.5 – 7.0

* - For solid medium 20.0g was added

3.4.2. Hydroxy Apatite Medium (Sperber, 1958a)

Soil extract*	-	100 ml
Glucose	-	2.5 g
Agar	-	20.0 g
Distilled water	-	900 ml
pH	-	7.0

* Five hundred grams of soil was mixed with 500 ml distilled water and steamed for 1h. The flask was kept undisturbed over night for settlement of mud and then filtered through coarse filter paper till the extract become clear.

Medium was distributed in 100 ml lots and sterilized. To the cooled 100 ml medium 5 ml of 10 per cent KH_2PO_4 and 10 ml of $CaCl_2$ were added just before plating and adjusted to pH to 7.0 with sterile 1 N NaOH.

3.4.3. Pikovskaya's Medium (Pikovskaya, 1948)

Glucose	-	10.0 g
Tricalcium phosphate	-	5.0 g
Ammonium sulphate	-	0.5 g
Potassium chloride	-	0.2 g
Magnesium sulphate	-	0.1 g
Manganese sulphate	-	Trace
Ferrous sulphate	-	Trace
Yeast extract	-	0.5 g
Agar	-	15.0 g
Distilled Water	-	1000 ml

3.4.4. Nutrient Agar/Nutrient Broth (Gordon *et al.*, 1973)

Peptone - 5.0 g
Beef extract - 3.0 g
Agar* - 20.0 g
Distilled water - 1000 ml
pH - 6.8-7.0

* Agar was avoided in nutrient broth.

3.4.5. Yeast Extract Glucose Agar

Yeast extract - 10.0 g
Glucose - 10.0 g
Agar - 15.0 g
Distilled water - 1000 ml
pH - 6.8-7.2

3.4.6. King's B Medium (KB) (King *et al.*, 1954)

Protease peptone - 20.0 g
Glycerol - 15.0 ml
Di potassium hydrogen phosphate - 2.5 g
Magnesium sulphate - 6.0 g
Agar - 18.0 g
Distilled Water - 1000 ml
pH - 7.2

3.4.7. Luria Bertaini medium (LB)

Tryptone	-	10.0 g
Yeast extract	-	5.0 g
Sodium chloride	-	10.0 g
Agar	-	18.0 g
Distilled Water	-	1000 ml
pH	-	7.2

3.4.8. Sporulating medium

Peptone	-	5.0 g
Beef extract	-	10.0 g
Manganese sulphate	-	0.005 g
Agar	-	18.0 g
Distilled water	-	1000 ml
pH	-	7.0

3.5. Collection of samples

Soil and feeder root samples were collected from different agroclimatic zones of the tea growing areas of southern India (Fig 1). Samples were collected from both seedlings and clones and 3 - 5 estates were covered in each zone for this purpose. The soil samples were air dried under shade and used for the isolation and enumeration of *Azospirillum* and PSB.

3.6. Isolation and enumeration of *Azospirillum*

For the isolation of *Azospirillum*, the rhizosphere soil samples were serially diluted up to 10^6 dilution using sterile distilled water. One milliliter of the soil diluent from each dilution was transferred to the tubes containing 10 ml of nitrogen free malic acid semi solid medium and kept for incubation for three days at 35±2ºC. The presence of *Azospirillum* was indicated by the formation of white characteristic undulating subsurface pellicle with the change of colour of the medium from yellowish green to blue. *Azospirillum* strains were brought to pure culture by streaking the cultures on plates containing nitrogen free malic acid medium and by repeated sub culturing. Pure cultures were maintained in nitrogen free malic acid agar slants.

Enumeration of *Azospirillum* in soil samples were carried out by most probable number method (MPN). One milliliter of successive dilutions of 10^4, 10^5 and 10^6 were transferred to test tubes containing nitrogen free malic acid semi solid medium. Five tubes were maintained for each dilution. The tubes were incubated at room temperature for 3 days. The positive tubes were counted and the population was calculated as per MPN tables (Cochran, 1950) and expressed as number of *Azospirillum* per g. dry weight of soil samples.

$$\textit{Azospirillum}\text{ population} = \frac{\text{MPN value x middle dilution}}{\text{Dry weight of the soil/root sample}}$$

3.7. Isolation and enumeration of PSB

Isolation and enumeration of PSB was carried out following dilution plate technique using hydroxy apatite medium. For the isolation PSB, the soil samples were serially diluted up to 10^6 dilution and plated on petriplates and incubated at 35±2ºC for seven days. At the end of incubation, PSB colonies were visually identified from the clear zone around the bacterial colony. The colonies were sub cultured, purified and maintained in nutrient agar slants.

3.8. Screening of *Azospirillum* strains

3.8.1. Preliminary screening

The *Azospirillum* strains were preliminarily screened by change in pH and colour of the medium. The selected *Azospirillum* strains were inoculated in the nitrogen free malic acid broth and incubated for three days. The colour and pH change were observed daily.

3.8.2. Nitrogen fixation by *Azospirillum* strains

Total nitrogen in the culture filtrate/soil was estimated by Kjeldhal method (AOAC, 1996). One milliliter of homogenized culture/0.1 gram of soil was transferred to Kjeldahl tube and added a Kjeltab, a pinch of sodium thiosulphate and 10 ml of salicylic acid - sulphuric acid mixture. This mixture was kept overnight and then digested in Kjeldhatherm apparatus by increasing the temperature up to 400ºC by stepwise increase of 50ºC at half an hour interval. At the end of digestion the mixture became clear liquid. The mixture was brought to room temperature and then distilled in a Gerhardt vapodest instrument by adding

40 ml of 40 per cent sodium hydroxide and the ammonia evolved was trapped in a 250 ml conical flask containing 10 ml of 0.07N hydrochloric acid. Afterwards the content was titrated against 0.07N sodium hydroxide using methyl red as indicator. The end point was the conversion of red colour to yellow. Total N was calculated as follows and represented on per cent basis.

$$\text{Total N (\%)} = \frac{\text{(Blank value-titrated value) x Normality of NaOH x 0.014}}{\text{sample weight}} \text{ x 100}$$

where, 0.014 - milli equivalent of nitrogen

The total nitrogen fixed by *Azospirillum* was calculated as follows and represented as mg/g of malate.

Nitrogen fixation (mg/g of malate) = Total N (%) x 10 x volume of culture in the flask

3.8.3. Acetylene reduction activity

The acetylene reduction activity of the *Azospirillum* isolates was determined following the method of Stewart *et al*. (1967). Semisolid nitrogen free malic acid medium was dispensed into 100 ml glass bottles in 50 ml lots and sterilized. The medium was inoculated with 1 m1 of culture and incubated at room temperature under static condition. After the incubation period, the cotton plugs were replaced with sterilized suba-seal rubber stoppers. The air inside the bottles was replaced with nitrogen by flushing through sterile syringe. Using another syringe, pure acetylene (C_2H_2) gas was injected at the rate of 1 per cent of the remaining gas phase, after withdrawing same volume of nitrogen without disturbing the pellicle, and incubated for 4h at room temperature. At the end of incubation, 1 m1 of the gas sample was withdrawn from the bottle and injected into a gas chromatograph fitted with FID system and 80-100 Poropak column) and the ethylene peak was recorded. The acetylene reduction activity was calculated using the formula.

$$\text{Area} = \frac{\text{n moles of ethylene x 60 x volume of gas x volume of the flask}}{\text{mg protein of the sample x 60 x sample volume}}$$

The acetylene reduction activity was expressed as n moles C_2H_4/mg protein/hour

3.9. Screening of PSB strains

3.9.1. Phosphate solubilization in solid medium

The PSB strains were inoculated in solid hydroxy apatite medium and incubated for 7 days. After incubation period the diameter of the halo region produced around the colonies was measured.

3.9.2. Change in pH of the medium

Selected PSB strains were grown in LB broth and inoculated 1 ml to Pikovskaya's broth. After incubation period the pH was measured at different period of growth.

3.9.3. Estimation of organic acids (Sperber, 1958b)

The organic acid produced by PSB strain was estimated in terms of total titrable acidity of the culture filtrate. PSB strains were inoculated in the Pikovaskaya's broth and allowed to grow for 7 days. After incubation period the culture was centrifuged and removed the cell biomass. Two milliliter of culture filtrate was taken in a flask and added few drops of phenolphthalein and titrated against 0.01 N of sodium hydroxide. The volume of alkali consumed by culture filtrate was the total titrable acidity of the culture filtrate. The total titrable acidity was expressed by ml of 0.01 N NaOH consumed.

3.9.4. Estimation of phosphatase activity (Eivazi and Tabatabai, 1977)

The PSB isolates were grown in Pikovaskaya's broth where TCP was replaced with organic source (p-glycerophosphate). The pH of the medium was adjusted to 5.2 and incubated at room temperature for 72 h over an environmental shaker. Ten milliliter of the broth culture was withdrawn, centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 5 ml of sterile distilled water. These cells were served as enzyme source. One milliliter of the sample was taken in a 50 ml Erlenmeyer flask, added 0.25 ml of toluene, 4 ml of modified universal buffer, 1 ml of p-nitrophenyl phosphate solution and swirled the flask for a few seconds to mix the content. The flask was tightly closed with the stopper and placed in an incubator at 35±2ºC for one hour. After incubation, the stopper was removed and added 1 ml of 0.5 M of calcium chloride and 4 ml of 0.5 M sodium hydroxide and swirled the flask again for a few seconds and filtered the content through a filter paper. The colour intensity was measured at 660 nm and calculated the p-nitrophenol content by reference to a calibration graph plotted from the results obtained with standard containg 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol. The phosphatase activity was expressed µ moles of PNP released/ml of filtrate/hour.

3.9.5. Estimation of available P

The available phosphorus in the soil/leaf/culture filtrate was estimated following the method of Olsen *et al.* (1954). The broth culture was centrifuged at 1000 rpm for 10 min and the clear supernatant was used for the estimation of available phosphorus.

In the case of soil, 5 g of air dried soil was extracted with extractant with a pinch of phosphate free activated charcoal and shake on a horizontal reciprocating shaker for 5 min and filtered through Whatman No.1 filter paper.

One milliliter of culture/soil filtrate was pipetted out into a 25 ml standard flask and added 10 ml of distilled water and shake well. Then 5 ml of freshly prepared ascorbic acid and ammonium molybdate solution were added and made up to 25 ml. Absorbance was measured at 882 nm after half an hour. A standard curve was prepared using 0, 1, 2, 3, 4 and 5 ml of 5 ppm standard P solution.

3.10. Identification of bacterial strains

The selected bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology (Krieg and Dobereiner, 1984).

3.10.1. Cell morphology

Bacterial smears were prepared on clean glass slides by using a loopful of culture at log phase. The smears were air-dried, heat fixed and stained with crystal violet. The cells were observed under light microscope with oil immersion objective to see the size and shape.

3.10.2 Gram staining

Bacterial cultures were uniformly spread on a clean glass slide and heat fixed by intermittent heating. A few drops of crystal violet was added and incubated for one minute, followed by flooded with Gram’s iodine solution. After one minute, the slide was washed under running tap water for a few seconds and blot dried. After de-staining in 95 per cent ethanol for 30 seconds, the preparation was washed in running tap water and counter stained with safranine stain for one minute. The stained preparation was washed in tap water, dried and examined under light microscope. The gram positive organisms were stained purple to blue black and the gram negatives stained red to pink.

3.10.3. Stains

i) Crystal violet solution

Crystal violet - 2.0 g
Ethanol - 20.0 ml

ii) Gram’s iodine solution

Iodine - 1.0 g
Potassium iodide - 2.0 g
Dist. water - 50.0 ml

iii) Safranine solution

Safranine O - 0.25 ml
Ethanol - 10.0 ml
Distilled water - 100 ml

3.10.4. Motility

Motility was tested by hanging drop method. Slides were prepared with log phase cultures and motility was observed under oil immersion.

3.10.5. Biotin requirement

The biotin requirement of the bacterial isolates was tested using semisolid nitrogen free malic acid medium prepared in two sets of tubes, one set of medium prepared with the addition of biotin (100 µg l^{-1}) and another without biotin. The medium was inoculated with bacterial cultures and incubated at room temperature. Uninoculated control was maintained for each set. The growth was observed by the change in colour from yellowish green to blue.

3.10.6. Denitrification

The dissimilation of nitrate by the bacterial isolates was tested following the method of Baldani and Dobereiner (1980). Nitrogen free malic acid medium (5 ml) supplemented with 5mM of ammonium nitrate and 1.5 per cent agar was taken in test tubes, sterilized and stab inoculated with the stock cultures. Nitrate reductase positive (nir+) isolates produced large quantities of gas, which caused shredding of agar block, which was not noticed with nir^- isolates.

3.10.7. Acid production

Glucose peptone broth (Krieg and Tarrand, 1978) was prepared and dispensed in 10 ml quantities in test tubes and sterilized. The bacterial cultures were inoculated at the rate of 1 ml per tube and incubated at room temperature. Acid production by the bacterial isolate was observed by change in colour of the medium from green to yellow.

3.10.8. Spore staining

Bacterial strains were grown in sporulating medium. Uniform smears with the PSB were made on a clean glass slide. It was allowed to air dry and heat fixed by intermittent heating. The slide was flooded with malachite green and allowed to react for 10 min. It was then washed in running tap water, counter stained with safranine stain and observed under a light microscope. The spores were appeared as dark green in a background of pink vegetative cells.

Stains

Malachite green (5 %)

Malachite green - 5.0 g
Distilled water - 100 ml

Safranine

Safranine - 0.25 ml
Ethanol - 10.0 ml
Distilled water - 100 ml

3.10.9. Catalase test

Selected strains were inoculated on LB agar plates and incubated at 28±2°C for 24 h. About three to four drops of 3 per cent H_2O_2 solution was allowed to flow over the culture. Formation of bubbles or effervescence indicated a positive result.

3.10.10. Oxidase test

A loopful of fresh bacterial cultures grown on LB agar medium was spotted on a Whatman No.1 filter paper. Few drops of freshly prepared aqueous solution of tetra methyl p-phenylene (1 %) were added. Appearance of a deep purple colour within 10 seconds indicated a positive result.

3.10.11. Indole production

Test tubes containing 5 ml of SIM agar medium were stab-inoculated with different bacterial strains and incubated for 24 h. Kovac's reagent was added to the cultures, production of a red coloured ring indicated the presence of indole compounds.

Diffused growth of bacterial strain is an indication that the strain is motile, while growth along the line of inoculation indicates that the strain is non-motile.

SIM agar medium

Peptone	-	30.0 g
Beef extract	-	3.0 g
Ferrous ammonium sulphate	-	0.2 g
Sodium thiosulphate	-	0.0025 g
Agar	-	12.0 g
Distilled water	-	1000 ml
pH	-	7.8

Kovac's reagent

p-dimethylaminobensaldehyde	-	5.0 g
Isoamyl alcohol	-	75 ml
Hydrochloric acid	-	25 ml

3.10.12. Methyl red and Voges - Pros-Kauer (MR-VP) test

About 5 ml of MR-VP broth was dispensed in test tubes and autoclaved. A loopful of 24 h old cultures of the bacteria were inoculated into the test tubes and incubated for 24 h. The broth was then divided into two parts: to one part methyl red indicator was added and to the other part Barrit's reagent was added. They were allowed to react for 15 min. The appearance of red colour indicates a positive result of methyl red test. Formation of a pink colour with the addition of few drops of Barrits reagent indicates a positive reaction for VP test. In both cases un-inoculated broth served as control.

MR-VP medium

Peptone	-	7.0 g
Dextrose	-	5.0 g
Di-potassium hydrogen phosphate	-	5.0 g
Distilled water	-	1000 ml
pH	-	6.9

Methyl red test

Methyl red	-	10.0 g
Ethanol	-	300 ml
Distilled water	-	1000 ml

Voges Pros-Kauer test

Barrits A

α-naphthol - 5.0 g

Ethanol - 95 ml

Barrits B

Potassium hydroxide - 40.0 g

Creatine - 0.3 g

Distilled water - 100 ml

3.10.13. Gelatin liquefaction

Nutrient agar supplemented with 1per cent gelatin was inoculated with the bacterial strains. After 7 days the solid medium was turned to liquefied indicated the positive result.

3.10.14. Starch hydrolysis

Bacterial cultures were streaked on starch agar plate and incubated at 28±2ºC for 4 days. The plates were then flooded with Gram’s iodine solution for 5 min. The iodine solution was then drained and the appearance of a hyaline halo around the bacterial growth indicated hydrolysis of starch. The plate without test strain was served as control.

Starch agar medium

Peptone - 10.0 g
Beef extract - 10.0 g
Soluble starch - 1.5 %
Sodium chloride - 5.0 g
Agar - 18.0 g
Distilled water - 1000 ml
pH - 7.0-7.2

3.10.15. Nitrate reduction test

Nitrite formation from nitrate was detected by growing the bacterial strains on Dye’s medium.

Dye’s medium

Di-potassium hydrogen phosphate - 0.5 g
Potassium di hydrogen phosphate - 0.5 g
Magnesium sulphate - 0.2 g
Sodium chloride - 5.0 g
Yeast extract - 2.0 g
Potassium nitrate - 1.0 g
Distilled water - 1000 ml
pH - 7.2

Test tubes containing 5 ml of Dye's medium was stab-inoculated with 24 hour old log phase cultures. After incubating for two days at room temperature, the cultures were tested for nitrite production by adding a few drops of sulphanilic acid and α-naphthylamine reagent. Formation of a distinct cherry red colour indicated the presence of nitrite.

Sulphanilic Acid	: 0.8 g of sulphanilic acid was dissolved in 100 ml of 5 N acetic acid.
α-naphthylamine regent	: 0.5 g of α -naphthylamine was dissolved in 100 ml of 5 N acetic acid.

3.10.16. Urease test

Christensen's urea agar medium was prepared without urea. Filter sterilized urea solution (40 %) was mixed with the autoclaved molten medium just before pouring into sterile petri plates. Fresh bacterial cultures were streaked on solidified medium and incubated for 48 h. Production of pink colour against a yellow background by the bacterial culture was indicated the positive result.

Christensen's urea agar

Peptone	-	1.0 g
Glucose	-	1.0 g
Sodium chloride	-	5.0 g
Potassium di hydrogen phosphate	-	2.0 g
Phenol red	-	0.12 g
Agar	-	18.0 g
Urea (40 %)	-	0.25 ml
Distilled water	-	1000 ml
pH	-	6.8

3.10.17. Citrate utilization

Simmon’s citrate agar medium was prepared, and made into slants. Fresh cultures of bacterial strains were streaked on the slants and incubated for 24 h at 28±2ºC. Citrate positive cultures turned blue, while citrate negatives remained green.

Simmons Citrate Agar

Ammonium phosphate	-	1.0 g
di-potaasium hydrogen phosphate	-	1.0 g
Sodium chloride	-	5.0 g
Magnesium sulphate	-	0.2 g
Agar	-	15.0 g
Bromothymol blue	-	0.08 g
Sodium citrate	-	2.0 g
pH	-	6.8 g

3.10.18. Arginine dehydrolase

This method was used for the differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. Thornley’s Agar medium was adjusted to faint pink colour (pH 7.2) and dispensed into 5 ml test tubes and autoclaved. The tubes were stab inoculated with 24 h old cultures and incubated at 28±2ºC for four days. Appearance of a distinct pink colour due to the presence of ammonia indicated arginine degradation. The tube without bacterial inoculation was served as control.

Thornley's Agar medium

Peptone	-	1.0 g
Sodium chloride	-	5.0 g
Di-potassium hydrogen phosphate	-	0.3 g
Phenol red	-	0.01 g
Arginine hydrochloride	-	10.0 g
Agar	-	3.0 g
Distilled water	-	1000 ml

3.10.19. Fluorescence test

This test had been used for identifying *Pseudomonas fluorescens* from other species of *Pseudomonas.* Freshly grown cultures were streaked on KB agar medium. The plates were incubated for 2 days at 28±2ºC and observed under UV light (355 nm) to see the fluorescence.

3.10.20. H_2S production

Test tubes containing YS broth were inoculated with fresh culture of bacterial strains. Strips of sterilized filter paper moistened with 10 per cent solution of filter sterilized neutral lead acetate were inserted into the test tubes in such a way that the lower end of the filter paper was kept about 5mm above the level of the medium. The blackening of the filter paper strips indicated the production of hydrogen sulphide. The medium without bacteria was served as control.

YS broth

Ammonium dihydrogen phosphate	-	0.5 g
Potassium di hydrogen phosphate	-	0.5 g
Magnesium sulphate	-	0.2 g
Sodium chloride	-	5.0 g
Yeast extract	-	5.0 g
Peptone	-	0.5 g
Distilled water	-	1000 ml

3.10.21. Pigmentation on BMS agar

The pigmentation produced by *Azospirillum* on BMS was tested by streaking the cultures on the medium, incubated and observed the pink colour pigmentation.

BMS Agar

Potato	-	200g
Malic acid	-	2.5 g
Potassium hydroxide	-	2.0 g
Raw Cane sugar	-	2.5 g
Vitamin solution	-	1.0 ml
Bromothymol blue	-	2 drops
Agar	-	15.0 g

3.10.22. Levan production for sucrose

Levan production from sucrose is a useful criterion for biotype differentiation in *Pseudomonas fluorescens.* Levan production was tested by using nutrient agar that contained 4 per cent sucrose. The levan formation by bacterial strains was elevated globous colonies from the surface of bacterial growth. The elevated growth sometimes may contain only mucous materials. The bacterial cells were checked by microscopically.

3.10.23. Growth in sodium chloride

The bacterial cultures were inoculated in nutrient broth containing different concentrations of sodium chloride (3 % and 7 %) and incubated at 28±2ºC and observed the growth.

3.11. Biodiversity study

3.11.1. Isolation of genomic DNA (Williams *et al.*, 1990)

Single colony of *Azospirillum and* PSB were transferred to their respective media and incubated on a rotary shaker at 150 rpm. The cultures were centrifuged at 7000 rpm for 7 min and the supernatant was discarded. To the pellet, 100 μl ice cold TEG and 200 μl of freshly prepared 2 per cent SDS were added and mixed by inverting the tube rapidly. Then 150 μl of 3 M potassium acetate was added and mixed gently for 10 seconds and centrifuged at 7000 rpm for 7 min. The aqueous phase was transferred to a fresh tube and added an equal volume of phenol: chloroform and centrifuged at 7000 rpm for 7 min. The aqueous phase was transferred to a fresh tube and the DNA was precipitated with two volume of ice-cold ethanol at room temperature for 10 min. Following precipitation, the samples were centrifuged at

10000 rpm for 10 min and the supernatant was discarded. The pellet was rinsed in 1 ml of 70 per cent ethanol and air-dried. The resulted DNA was dissolved in 50µl of water. The quantity and quality of the extracted DNA was checked spectrophotometrically and also by 0.8 per cent of agarose gel electrophoresis stained with ethidium bromide.

3.11.2. RAPD Analysis

RAPD analysis was carried out through PCR amplification of total DNA using a Minicycler, M J Research (USA). The following random primers (OPERON) were used for amplification for *Azospirillum* strains.

1. OPA 2 (5'-TGCCGAGCTG-3')
2. OPA 3 (5'-AGTCAGCCAC-3')
3. OPA 4 (5'-AATCGGGCTG-3')
4. OPA 5 (5'-AGGGGTCTTG-3')
5. OPA 6 (5'-GGTCCCTGAC-3')
6. OPA 7 (5'-GAAACGGGTG-3')
7. OPD 20 (5'-ACCCGGTCAC-3')

The RAPD profile of the ten each of *Azospirillum* and PSB strains, along with the type strain using the primers OPA2, 3, 4, 5, 6, 7 and OPD 20 was studied.

PCR amplification reaction was performed (Williams *et al.*, 1990) using 5µl of 50 ng DNA sample, 3µl of 50 pmol primer, 2µl of 0.2mM dNTPs, 2µl of PCR buffer 0.3µl of *Tag* polymerase enzyme and 7.7 µl water. The amplification reaction was performed following thermal condition; initial denaturation for 4 min at 94ºC followed by further denaturation for

2 min at 72ºC, annealing was followed for 1 min at 38ºC and 2min extension at 72ºC and final extension at 72ºC for 10 min. Amplification products were analysed on 1.2 per cent agarose gel in 1X TBE buffer.

3.11.3. Dendrogram

The separated amplified DNA banding pattern was scored manually based on the presence/absence of bands. A dendrogram was constructed based on a similarity index by the unweight pair group means method according to Sneath and Sokal (1973).

3.12. *In vitro* tests

3.12.1. pH tolerance

The pH tolerance of selected strains of *Azospirillum* and PSB was tested in yeast extract glucose agar medium prepared at pH range of 4.0 to 9.0 with an increment of 0.5 units. The cultures were streaked on the medium and incubated at 35±2ºC. After 3 days the growth of the *Azospirillum* and PSB strains were observed.

3.12.2. Temperature tolerance

The bacterial strains were streaked on LB medium and kept at different temperature (5, 15, 28, 35 and 50ºC) and recorded the growth.

3.12.3. Antibiotic resistance

The antibiotic resistance was tested on yeast extract glucose agar (Allen, 1953) incorporated with various antibiotics like penicillin (50 – 300 ppm), chloramphenicol (50 -300 ppm) and streptomycin (50 - 300 ppm). The antibiotic solution was prepared, filter sterilized and added to the medium before plating. The plates were streaked with selected strains of *Azospirillum* and PSB, incubated at 35±2ºC and observed the growth.

3.12.4. Pesticide tolerance

For this study, the pesticide Dicofol and Ethion were selected. Dicofol (1000 - 5000 ppm) and Ethion (1000 - 5000 ppm) were prepared and filter sterilized and incorporated with yeast glucose agar medium. The strains were streaked on it and incubated at 35±2ºC. The pesticide tolerance was observed by nature of growth of the strains.

3.12.5. Fungicide resistance

The popular fungicides Copper Oxy Chloride (COC) and Tilt were tested for the fungicide tolerance of selected strains of *Azospirillum* and PSB. The stock solution COC (50 - 500ppm) and Tilt (1000 - 5000 ppm) were prepared and incorporated with yeast glucose agar. The bacterial strains were streaked on the medium and kept at 35±2ºC temperature and observed for their growth.

In all these cases, the growth was graded as no growth (-), poor growth (+), medium growth (++), good growth (+++) and very good growth (++++) (Plate 2).

3.12.6. Impact of carbon sources on N fixation and P solubilization

To find out the impact of different carbon sources on N fixation, the sources were incorporated into *Azospirillum* growing culture medium and estimated the acetylene reduction activity.

In order to study the effect of different types of carbon sources on P solubilization, the selected PSB strains were inoculated in Pikovskaya's broth with glucose, fructose, maltose and starch as carbon sources. The efficiency of P solubilization was found out by estimating the solubilized phosphorus by Olsen *et al.* (1954) method.

3.12.7. Impact of nitrogen sources on N fixation and P solubilization

Different types of nitrogen sources such as ammonium chloride, potassium nitrate, ammonium nitrate, ammonium sulphate and urea were incorporated and found out their impact on N fixation. Effect of nitrogen sources on P solubilization was found out by inoculation of PSB strain with different types of nitrogen sources in the Pikovskaya's broth and estimated the solubilized phosphorus in the culture medium.

3.12.8. Effect of different phosphate sources on P solubilization

The efficiency of P solubilization by PSB strains was estimated with different phosphate sources *ie.* $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Mg_3(PO_4)_2$. The broth culture medium amended with different phosphate sources were inoculated with PSB strains and after a period of incubation, available phosphorus in the culture medium was estimated.

3.12.9. Utilization of carbon, nitrogen, amino acid and vitamin compounds

Utilization of different carbon, nitrogen, amino acid and vitamin compounds by *Azospirillum* and PSB isolates were estimated in LB broth. Filter sterilized carbon, nitrogen, amino acid and vitamin sources were inoculated aseptically into the sterile medium at 1 per cent level. The *Azospirillum* and PSB cultures were inoculated at the rate of 1 ml and incubated at 35±2°C. The growth was observed by measuring the absorbance at 560 nm.

3.12.10. Estimation of ammonium excretion

Ammonium excretion was estimated following the method of Narula *et al.* (1981). The *Azospirillum* strains were inoculated in 30 ml of malate broth in 150 ml conical flask for 7 days at room temperature. The culture broth was withdrawn and centrifuged at 10000 rpm for 30 min and ammonia released in supernatant was determined with ammonium sulphate as the standard.

3.12.11. Estimation of poly -β- hydroxy butyrate (PHB)

The PHB production by the *Azospirillum* and PSB strains were determined *in vitro* following the method of Zevenhuizen (1981). *Azospirillum* cells were harvested by centrifugation at 8000 rpm for 20 min at 4ºC. The pellet was suspended with sterilized distilled water and used for PHB estimation. To 1 ml of cell suspension, 1 ml of 2 N hydrochloric acid was added and digested at 100ºC for 2 h. The content was cooled down and extracted twice with chloroform. The chloroform was evaporated on boiling water bath and to the sediment, 5 ml of concentrated sulphuric acid was added. The content was heated at 100ºC for 10 min in a water bath. After cooling, the absorbance of the content was read at 235 nm in a spectrophotometer against concentrated sulphuric acid as blank.

3.12.12. Estimation of siderophore (Reeves *et al*., 1983)

Azospirillum and PSB cultures were grown in their respective media in 250 ml conical flask in a shaker for 72 h. The culture broth was centrifuged at 8000 rpm for 2 min and the pellet was rejected. Twenty milliliter of supernatant was taken in a separating funnel and adjusted the pH to 2.0 with 0.1 N HCl. To this, 20 ml of ethyl acetate was added and shaken well. The organic phase was carefully collected and the extraction was repeated atleast twice. Five milliliter of Hathway's reagent (1 ml of 0.1M ferric chloride + 1 ml of 0.1 M potassium ferricynade in 98 ml of distilled water) was added to 5 ml of the sample, shaken well and the absorbance was read at 700 nm in a spectrophotometer with dihydroxy phenol/salicylic acid as standard.

3.12.13. Estimation of exopolysaccharide (Dische, 1962)

Bacterial strains were inoculated in basal medium supplemented with 5 per cent sucrose and incubated for 120 h at 37ºC in a rotary shaker. Culture broth was centrifuged at 40000 g for 30 min and extracted for exopolysaccharides (EPS) by adding 3 volume of chilled acetone to 1 volume of extract. The EPS was measured by phenol-sulphuric acid method.

3.12.14. Estimation of NRA

The NRA was estimated in both soil and leaf samples following the *in vivo* assay method (Sarmah *et al*., 1987). The sliced leaf sample (300 mg) or soil sample (0.5 g) was suspended in a Thunburg tube containing 5 ml of medium comprising 0.1M phosphate buffer having pH of 7.5, 0.02 M KNO_3, 6 per cent polyclar AT and two drops of chloramphenicol. The tubes were evacuated for one minute and then the vacuum was released. The tubes were incubated at 30°C in the dark for 4 hrs. The reaction mixture was filtered through Whatman No. 1 filter paper. One milliliter of the medium was withdrawn and 1 ml of 1 per cent sulphanilamide and 1 ml of 0.02 per cent N-1-naphthyl ethylene diamine dihydrochloride were added to produce a pink diazo complex with the nitrite formed. Absorbance was read at 540 nm.

3.12.15. Estimation of indole acetic acid

The IAA production by the *Azospirillum* and PSB isolates *in vitro* condition was determined following the method of Tien *et al.* (1979). The *Azospirillum* strains were grown in nitrogen free malate broth without bromothymol blue and PSB strains in Pikovaskaya’s broth. Freshly prepared filter sterilized solution of L-tryptophan was added to the flask to a final concentration 100 µg per ml and suitable control was maintained. One milliliter of the inoculum was added into each flask and incubated at 30ºC for 7 days in dark.

After the incubation period, the culture was centrifuged at 6000 rpm to remove the bacterial cells and the supernatant was brought to pH 2.8 with 1 N HCl. Fifty milliliter of the acidified supernatant was taken in 100 ml conical flask and to it, equal volume of diethyl ether was added and incubated in dark for 4 h. lAA extraction was done at 4°C in a separating funnel using diethyl ether. The aqueous phase was discarded and the solvent phase was pooled and evaporated to dryness. To 5 ml of the methanol extract, 1.0 m1 of distilled water and 4 m1 of salper's reagent were added and incubated in dark for 1 h. The intensity of pink colour was read at 535 nm in a spectrophotometer (Specord S 100). From the standard curve prepared with known concentration of standard 1AA, the quantity of lAA in the culture filtrate of *Azospirillum* and PSB was determined.

3.12.16. Estimation of Gibberellins (Mahadevan and Sridhar, 1982)

The culture filtrate of *Azospirillum* and PSB were prepared as above. Fifteen milliliter of culture filtrate was taken in a test tube and to it 2 ml of zinc acetate was added and incubated for two min. To this, 2 ml of potassium ferrocynide solution was added and the mixture was centrifuged at 10000 rpm for 10 min. To 5 ml of the supernatant, 5 ml of 30 per cent hydrochloric acid was added and the mixture was incubated for 1h. The blank was prepared with 5 per cent hydrochloric acid and the absorbance was measured at 254 nm in a spectrophotometer.

3.12.17. Estimation of protein (Lowry *et al.*, 1951)

One milliliter of the homogenized cell culture/0.5 ml of leaf extract was taken in a test tube and the volume was made up to 5 ml. Five milliliter of alkaline copper solution was added to each tube mixed thoroughly and allowed to stand for 30 min. The intensity of blue colour developed was read at 660 nm against appropriate blank. A standard graph for protein prepared by using different concentrations of bovine serum albumin (BSA) was used for the estimation of protein content of *Azospirillum* isolates.

3.12.18. Estimation of total chlorophyll (Wellburn, 1994)

About 0.5 g of the sample was taken in mortar and ground well with 1 ml of methanol. Then another 4 ml methanol was added and ground well. The filtrate was filtered through the buckner apparatus and the mortar was washed with 5 ml methanol and the extract was poured into 50 ml standard measuring flask and made up to the mark with methanol. About 2 ml of the filtrate was pipetted out into a test tube and 8 ml of methanol was added.

The absorbance was recorded at 470, 653 and 666 nm. The chlorophyll content was calculated using the formula,

Chlorophyll a (mg/L) = (15.65 x A_{666}) – (7.34 x A_{653})

Chlorophyll b (mg/L) = (27.05 x A_{653}) – (11.2 x A_{666})

To convert mg/g = (GR x 50 x 10)/ (Wt. x 2)

3.13. Yield

The green leaf yield was recorded at every plucking round and converted to made tea (MT) kg per hectare by following formula,

$$\text{Made Tea} = \frac{\text{Green leaf yield (kg) x bush population per hectare x 0.225}}{\text{Number of bushes in the experimental plot}}$$

0.225- conversion factor for green leaf to made tea (Out turn)

3.14. Mass multiplication of bioinoculants

3.14.1. Growth Kinetics

Growth kinetics was studied under three different conditions *ie.,* static (Plate 3A), shaking (Plate 3B) and in the fermentor (Plate 3C). The bacterial strains were cultured in liquid medium and no fresh medium was provided during incubation and observed the population level for both the organisms. The population was plotted against incubation period and developed the growth curve.

3.14.2. Mass multiplication

Azospirillum and PSB isolates were multiplied in the fermentor (BIOFLO 2000) with nitrogen free malic acid broth and nutrient broth medium respectively. The broth culture was mixed with sterilized carrier materials. The viable count in the inoculum was kept as $2x10^9$/ml before mixing with carrier materials.

3.14.3. Carrier material

Various organic materials and agricultural wastes such as composted coir pith, lignite, organic manure, vermicompost and vermiculite were used for the mass multiplication of *Azospirillum* and PSB. The carrier materials were sterilized, sieved and maintained proper water content in the carrier materials. The bacterial strains were mass multiplied in fermentor and liquid culture was mixed with the carrier materials and used for nursery and field experiments.

3.14.4. Shelf life of bioinoculants

The shelf life of bioinoculants in different carrier materials was studied in a laboratory experiment. The biopreperation in different carrier materials were packed in polythene bags and stored in dust free cup board. The population load was checked every month for one year.

3.15. Nursery Experiment

Nursery experiments were conducted in UPASI TRF tea nursery. Nursery sleeves of 200 gauge and 10 x 30 cm size were filled with soil and sand mixture (approximately 1.5 kg). Before filling the soil into sleeves, the soil pH (4.8) and EC (0.029) were adjusted. The bottom 20 cm layer of sleeves was filled with jungle soil-sand mixture (growing zone) and top 10 cm was filled with red soil-sand mixture (rooting zone). The biofertilizers were applied in growing zone/rooting zone based on the nature of experiments. VP plants were raised through one leaf cuttings put out in nursery sleeves. Seedlings were raised by sowing the seeds in sand bed and they were transferred to nursery sleeves when the seed coat was cracked.

Biometric parameters such as shoot length, root length and dry weight and biochemical parameters such as total nitrogen, phosphorus, NRA, growth regulators *etc.*, were recorded with various objectives under nursery conditions. In addition to this, population level also estimates for *Azospirillum* and PSB in nursery soil as well as in roots.

3.16. Field Experiments

Field experiments were conducted with selected strains of *Azospirillum* and PSB with various objectives to prove their potential in supplementing nutrients to tea. Experiments were conducted in Anamallais as well as other agroclimatic zones like High Range and Nilgri - Wayanad with region specific strains. In multilocational trails in addition to the regional strains, common strains were also included. In all the field experiments, the biofertilizers were incorporated to the soil with composted coir pith as the carrier material. Nitrogen fertilizers were reduced by 20 and 40 % and phosphatic fertilizers by 50 % on the recommended dosage. The rock phosphate was added only on the alternate years. The number of treatments and replicates were fixed according to the type of experiments. The bioformulations were incorporated near to the root zone by punching holes with a crowbar. *Azospirillum* and PSB were put in separate holes with soil as filler.

3.17. Organic Farming

The organic manures such as compost and neemcake were applied in the staggered trenches. The dimension of trenches was 2 x 0.3 x 0.45 m and 2 m distances between two trenches. The trenches were taken across the slop in every row. The phosphatic fertilizer was applied by placement at 10 - 20 cm depth in holes made by crowbar on both sides of tea bushes on the upper side of the slope. The potassium source was given by addition of wood ash during dry period by broadcasting. The biofertilizers (*Azospirillum* and PSB) were incorporated by placement.

RESULTS

4.1. Chapter I

ISOLATION AND SELECTION OF N-FIXING AND P-SOLUBILIZING BACTERIA

4.1.1. Isolation of N-fixing and P-solubilizing bacteria

Totally 236 *Azospirillum* and 114 PSB strains were isolated from soil samples collected from different agro climatic zones of the tea plantations of southern India and brought to pure culture (Table 1; Plate 4A & 4B).

Enumeration of these organisms showed that the population level of *Azospirillum* was higher than phosphobacteria in tea soils of all districts, except Vandiperiyar. .*Azospirillum* population was higher in Anamallais followed by Nilgiri - Wayanad and least in Karnataka. In the case of phosphobacteria, the population level was highest in Vandiperiyar and least in Wayanad (Table 1).

4.1.2. Selection of N-fixing and P-solubilizing bacteria

To select the best strains of *Azospirillum* and PSB, the bacterial strains were screened *in vitro*. The *Azospirillum* strains were preliminarily screened by noting the change in colour and pH of the medium. Various strains differed in their ability in changing the colour intensity of the medium (Table 2). There was a direct correlation between the intensity of bluish colour and the alkalinity of the medium. The strains those changed the colour of the medium and pH drastically were selected for further studies.

The *Azospirillum* strains thus selected were further examined for fixation of atmospheric nitrogen *in vitro* in the N-free semisolid malate medium. Among ten isolates, M 2 (75 mg/g of malate) and M 4 (74 mg/g of malate) were superior in fixing atmospheric N followed by W 5 and N 1. The isolates C 26, G16 and CT 8 were least in their potential. The strains from Anamallais and High Range had higher potential of nitrogen fixation (Table 3).

Acetylene reduction assay (ARA) of these strains ranged from 42.3 (M 4) to 157.3 (CT 8) n moles of C_2H_2 produced/mg protein/hr. Among the ten selected strains, CT 8 possessed highest potential of acetylene reduction followed by AN 45. The isolates K 4, N 1, M 2 and AB possessed more or less same level of acetylene reduction ability. The acetylene reduction potential of C 26 and M 4 was comparatively low (Table 3).

The PSB strains were screened *in vitro* by measuring the P solubilization zone in solid medium, determining pH change of the medium and estimating phosphatase activity, organic acids production and available P. Among 114 strains, 77 were selected based on the P-solubilization. Testing of these strains for P solubilization revealed that among these 10 strains (ANP 24, CP 29, GP 7, WP 11, MP 17, CTP 19, KP 24, NP 9, MP 5 and PB) had higher potential (Table 4). These ten efficient strains were selected for further studies.

Among these ten strains, PB was found to be superior in forming halo zone of P solubilization followed by MP 5 (Table 5). All the strains brought down the pH of the culture medium. However, the maximum reduction was noticed with ANP 24 followed by MP 5, PB and CTP 19. Estimation of titrable acidity indicated that the organic acid production was highest with strains PB and WP 11 followed by MP 5. Strains MP 17, KP 24, CP 29, GP 7 and NP 9 were poor in organic acid production (Table 5).

Estimation of phosphatase activity of selected strains of phosphobacteria indicated that the strain PB exhibited higher phosphatase activity followed by WP 11 and MP 17 was the least (Table 5). The P solubilization potential of selected strains of PSB was tested *in vitro* by estimating available phosphorus in the culture medium. The results indicated a wide variation in the phosphate solubilization capacity of different strains (Table 5). Among these, PB released more phosphorus (46.5 ppm) to the medium followed by ANP 24 (44.0 ppm). Thus it is clear that among the ten strains, PB has higher phosphate solubilization potential.

4.1.3. Identification of *Azospirillum*

The selected strains of *Azospirillum* were identified up to species level based on biochemical tests following Bergey’s Manual of Determinative Bacteriology. These isolates were found to be identical with *Azospirillum* sp. forming typical white, dense and undulating fine pellicle in nitrogen free semi-solid medium, Gram negative, spiral movement, 0.8 - 1.0 µm in diameter and 2.0 - 3.5 µm in length (Plate 5A). Further more, the presence of PHB granules gave more evidence to confirm the identity of the *Azospirillum* isolates.

Among the ten strains, C 26, G 16, W 5, M 2, M 4, K 4, CT 8 and N 1 were found to produce acid from glucose peptone medium, required biotin for their growth and fixed the atmospheric nitrogen under microaerophilic condition, characteristics of *Azospirillum lipoferum.*

A. lipoferum is vibriod, Gram negative, 1.0 µm in diameter and 2.0-3.5 µm in length, microaerophilic and motile and have poly β-hydroxy butyrate in their cells. The colonies were pink in colour in BMS agar. *A. lipoferum* required biotin for their growth and acidification in peptone based glucose medium. They were able to produce acid from glucose and fructose anaerobically. In semisolid nitrogen free malate medium, the cells became wider. It was positive to oxidase, catalase, phosphatase, nitrate reduction, urease, esculin hydrolysis and acid production from fructose, galactose, arabinose *etc*. *A. lipoferum* was negative to gelatin hydrolysis, indole production, starch hydrolysis, VP test and acid production from rhamnose, sucrose, lactose *etc.* (Table 6).

The rest of the isolates, AN 45 and AB failed to produce acid in glucose peptone medium and did not require biotin for their growth and fixed the atmospheric nitrogen under microaerophilic condition (Table 6). They were identified as *A. brasilense.*

Azospirillum brasilense is vibriod, 1.2 µm in diameter and 2.0 - 3.7 µm in length, Gram negative, microaerophilic and motile. Colonies were pink in colour in BMS agar. Biotin was not required for their growth and were not able to produce acid in peptone based glucose medium. They were positive to oxidase, catalase, phosphatase, nitrate reduction, urease, acid production from fructose, galactose, arabinose *etc.* while negative to gelatin hydrolysis, indole production, starch hydrolysis, growth in 3 per cent NaCl, VP test and acid production from mannitol, sorbitol, ribose *etc.* and unable to use α-ketoglutarate, mannitol, ribose *etc.* In semisolid nitrogen free malate medium, the cells were not wider (Table 6).

4.1.4. Identification of PSB

Based on the biochemical tests, the PSB strains were identified up to species level. The results of various biochemical tests for ten PSB isolates were summarized in Table 7. The isolates ANP 24, MP 17, CTP 19, KP 24, MP 5, PB and NP 9 were identical as *Pseudomonas putida,* while CP 29 and GP 7 were identified as *P. fluorescens* and WP 11 as *Bacillus megaterium*.

Pseudomonas putida: Cells rod shaped, 0.5 - 0.8 x 1.0 - 4.0 µm in size. They were Gram negative and motile and growth was strictly aerobic and spores were absent in all pseudomonads. On YDC agar, the colonies were cream coloured. *P. putida* were positive to oxidase, catalase, arginine dihydrolase, acid from glucose and growth on citrate agar and negative to growth at 41ºC, gelatin hydrolysis, starch hydrolysis and denitrification.

Pseudomonas fluorescens: Cells rod shaped and 0.5 - 1.0 x 1.5 - 5.0 µm in size (Plate 5B). They were Gram negative, motile and growth was strictly aerobic and not producing spores. On King’s B medium, they produced fluorescein, a water soluble pigment. On YDC agar and NY agar, the colonies were cream coloured. Cells positive to oxidase, catalase, lipase activity, arginine dihydrolase, gelatin hydrolysis, acid from glucose, and urease and negative to growth at 41ºC and starch hydrolysis.

Bacillus megaterium: Rod shaped, cells 0.5 - 1.5 x 1.5 - 6.0 µm in size. Growth strictly aerobic and produced endospores. Cells were Gram positive and motile in nature. Growth in glucose agar was mucoid. They were positive to catalase, acid from glucose, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, citrate utilization nitrate reduction and growth at pH 6-8 on nutrient broth and negative to anaerobic growth, gas from glucose, VP test, indole production and growth with lysozyme (Plate 6,7,8,9 and 10).

4.1.5. Biodiversity of *Azospirillum* and PSB

The selected strains from various agroclimatic zones were compared at molecular level by Random Amplified Polymorphic DNA (RAPD) analysis and constructed a dendrogram based on their similarity/dissimilarity coefficient. Ten strains each of *Azospirillum* and PSB strains were selected for genetic diversity study. The RAPD profile of the ten *Azospirillum* isolates, along with the type strain (NCIM 2907) using the primers OPA2, 3, 4, 7 and OPD 20 is given in Fig 2a. *Azospirillum* strains were grouped into three major clusters separated at a similarity coefficient ranging from 0.5 to 0.8. The similarity co-efficient was 0.60 for first cluster, 0.56 for second cluster and 0.50 for third cluster. Accordingly, the Anamallais strain AN 45 (1), Karnataka strain K 4 (5), High Range strain M 2 (7), Nelliampathy N 1 (8) and Vadiperiyar strain CT 8 (9) form the first group, Gudalur G 16 (4), and Wayanad W 5 (10) strains form the second group and Anamallais strain AB (2), Nilgiri strain C 26 (3) and High Range strain M 4 (6) form third group Fig 2a.

The RAPD profile of the ten PSB strains along with the type strain using the primers OPA2, 3, 4, 5 and 6 was given in Fig 2. PSB strains were grouped into two major clusters separated at a similarity coefficient ranging from 0.2 to 0.6. The similarity co-efficient was 0.25 for first cluster and 0.20 for second cluster. Accordingly, the Anamallais strains ANP 24 (1), PB (2), Nilgiri strain CP 29 (3), Karnataka strain KP 24 (5), High Range strains MP 17 (6), MP 5 (7), Vadiperiyar strain VP 19 (9) and Wayanad strain WP 11 (10) form the first group, Gudalur GP 7 (4) and Nelliampathy strains NP 9 (8)form the second group Fig 2b.

4.2. Chapter II

BIOLOGY AND ECOLOGY OF SELECTED STRAINS OF *AZOSPIRILLUM* AND P-SOLUBILIZING BACTERIA

4.2.1. Biology

4.2.1.1. Selection of media

Based on the nature of growth of *Azospirillum* and PSB, the culture media were selected. Okon's medium was found as the best for the growth of *Azospirillum* and Pikovskaya's medium for PSB. The growth of *Azospirillum* and PSB was significantly high in Okon's medium and Pikovskaya's medium respectively on 3rd day itself (Table 8 and 8a).

4.2.1.2. Growth kinetics

Growth kinetics of *Azospirillum* and PSB was studied by growing them in liquid medium under three conditions *viz.,* static culture, shake culture and in fermentor. The *Azospirillum* culture grown in fermentor attained higher population level on 3rd day, shake culture on 5th day and static culture on 6th day. In fermentor, the culture growth started declining on 7th day whereas in shake culture on 8th day and static culture on 9th day (Fig 3A).

In the case of PSB, the culture grown in fermentor attained higher population level on 3rd day, shake culture on 5th day and static culture on 7th day. In fermentor, the culture growth started declining on 7th day whereas in shake culture at 8th day and static culture at 9th day (Fig 3B).

4.2.1.3. Influence of pH on growth

The optimum pH for the growth of *Azospirillum* and PSB was ranged from 5.0 to 7.5. However, *Azospirillum* strains G 16, M 4, CT 8 and AB were able to grow at pH 4.5. In the case of PSB, except for CP 29 and KP 24 all others were able to grow at pH 4.5 (Tables 9 and 10).

4.2.1.4. Influence of temperature on growth

All the bacterial strains preferred temperature ranging from 15ºC to 35ºC above and below which the growth was retarded. *Azospirillum* strains G 16, K 4, M 4 and W 5 and PSB strain GP 7 could grow well at temperature 5ºC. None of them grew at -5ºC (Table 11).

4.2.1.5. Antibiotic resistance

All the *Azospirillum* strains showed tolerance up to 300 ppm of penicillin, 200 ppm of chloramphenicol and 250 ppm of streptomycin. In the case of PSB the tolerance level was 200 ppm of penicillin, 300 ppm for chloramphenicol and 300 ppm for streptomycin (Tables 12 and 13).

4.2.1.6. Pesticide resistance

All the bacterial strains could tolerate up to 3000 ppm of Dicofol and 2000 ppm of Ethion. However, MP 5 and PB could tolerate up to 3000 ppm of Ethion (Tables 14 and 15).

4.2.1.7. Fungicide resistance

The selected strains of *Azospirillum* and phosphobacteria were tolerant up to 200 ppm of COC and 5000 ppm for Tilt. Among these two fungicides, the tolerance level was low with COC compared to Tilt (Tables 16 and 17).

4.2.1.8. Utilization of carbon compounds

Azospirillum and PSB strains utilized various compounds as carbon source. The preferential carbon sources varied from strain to strain. Most of the *Azospirillum* strains preferred trehalose, melibiose, xylose, rhamnose, and adonitol as carbon source. On the other hand, fructose, maltose, arabinose, inulin, inositol and salicin were moderately utilized, while starch and manodione were found to be poor sources (Table 18).

Among the organic compounds, malate served as the best carbon source followed by succinate. Aspartate and citrate were poorly utilized and other compounds were utilized moderately (Table 18a).

All the PSB isolate except ANP 24 utilized manodione, starch, rhamnose and inositol as carbon source. Fructose, maltose, arabinose, dulcitol, inulin, melibiose, salicin, xylose and trehalose were utilized moderately. Adonitol was a poor source (Table 19). Among organic compounds succinate was the best carbon source. Lactate, propionate, acetate and malate were utilized only moderately (Table 19a).

4.2.1.9. Utilization of nitrogenous compounds

It was observed that *Azospirillum* isolates poorly utilized nitrogenous compounds such as ammonium sulphate, ammonium nitrate, ammonium tartrate, ammonium chloride and urea (Table 20).

All the nitrogen compounds supported the growth of PSB strains. Ammonium sulphate, ammonium nitrate and potassium nitrate were good sources and others were utilized moderately (Table 20a).

4.2.1.10. Utilization of amino acids

All the amino acids supported the growth of *Azospirillum* and PSB strains. Amino acids like tyrosine, tryptophan and methionine were the preferred sources by *Azospirillum* compared to others. Among the strains, K 4 and AB utilized all amino acids except phenylalanine, cystine and glutamic acids, but AN 45 and C 26 poorly utilized all the amino acids (Table 21).

On the other hand, PSB preferred leucine, cystine, serine, glycine and praline, but for ANP 24 and CP 29. Among the strains, MP 17 utilized maximum number of amino acids and ANP 24 and CP 29 strains poorly utilized almost all amino acids (Table 22).

4.2.1.11. Utilization of vitamins

All *Azospirillum* strains readily utilized nicotinic acid, Vitamin B1 and folic acid. While, Vitamin A, B2, B6, B12, C, D3 and K were utilized moderately (Table 23).

In the case of PSB strains, Vitamin B12, C and nicotinic acid were the preferred vitamins (Table 24).

4.2.1.12. Plant growth promoting substances

The beneficial microorganisms produce plant growth hormones such as IAA, GA3 *etc.*, in the growth medium. In general, *Azospirillum* produced more IAA than PSB. Both the group of organisms were comparable on their potential in producing GA3 (Table 25). Among the selected *Azospirillum* strains, AB produced more IAA followed by G 16 and AN 45 and least by K 4. The strain G 16 produced more GA3 followed by K 4 and AB and least by N 1 (Table 25).

In the case of PSB, MP 17 and MP 5 produced higher level of IAA followed by PB and least by ANP 24. The strain PB produced more GA 3 followed by KP 24 and WP 11 and least by GP 7 (Table 25).

4.2.1.13. Ammonium excretion and PHB production

The ammonia excretion by *Azospirillum* varied in different strains. Among them, M 2 excreted more ammonia (32.8ppm) followed by AN 45 (29.6ppm) and least by W 5 (19.4ppm). The ammonium excretion in most of the strains reached maximum at 12th day and then declined (Fig 4).

A wide variety of microorganisms were known to produce intracellular energy and carbon storage compound known as poly -β- hydroxy butyrate (PHB). A wide variation was observed in the production of PHB by *Azospirillum*. Strain M 2 produced higher level of PHB and ammonia, while it was low with strain M 4 (Table 26).

4.2.1.14. Production of siderophore and exopolysaccharide (EPS)

The PSB strains produced more siderophore and EPS compared to *Azospirillum* (Table 27). Among *Azospirillum* strains, M 2 produced more siderophore followed by AB and AN 45 and least by G 16. In the case of PSB, strain PB produced maximum siderophore followed by MP 5 and ANP 24 and least by GP 7 (Table 27).

Among the *Azospirillum,* K 4 produced maximum EPS (47.4 ppm) followed by M 2 (44.2 ppm) and AN 45 (44.0 ppm). Among PSB, it was maximum with MP 5 (51.6 ppm) and least in WP 11 (32.1 ppm) (Table 27).

4.2.2. Factors influencing nitrogenase activity

4.2.2.1. Impact of carbon and nitrogen sources on nitrogenase activity

External application of nitrogen and carbon sources greatly influenced the nitrogenase activity in the culture. Carbon sources had a positive impact (Fig 5), while nitrogen sources had a negative impact (Fig 6). Among the carbon sources malate supported the nitrogenase activity to the maximum followed by maltose. On the other hand, addition of nitrogen sources decreased the nitrogenase activity to varying level. The suppressive effect was maximum with urea followed by ammonium sulphate (Fig 6).

4.2.2.2. Impact of abiotic factors on nitrogenase activity

The abiotic factors such as pH and temperature influenced the nitrogenase activity. The optimum pH range for nitrogen fixation was 4.5 - 6.5, below and above this the activity was reduced (Fig 7).

Optimum temperature for nitrogenase activity was found to be 20 - 30ºC. The organism was able to tolerate up to 40°C above which the activity was retarded drastically. Likewise, below 10°C also the activity was reduced drastically. At 20ºC the quantity of C_2H_2 produced was 95.6 n mole/mg protein/hr which was reduced to 45 n mole/mg protein/hr at 10°C and at 5°C the quantity was further reduced to 19.7 n mole/mg protein/h (Fig 8).

4.2.2.3. Effect of incubation period on nitrogenase activity

Testing for nitrogenase activity *in vitro* at different incubation period indicated that the activity gradually increased up to 3rd day of incubation and then started decling (Fig 9).

4.2.2.4. Effect of nature of medium on nitrogenase activity

The nature of culture media greatly influenced the activity of nitrogenase enzyme *in vitro.* It was found that the semisolid medium was the best for nitrogenase activity compared to solid and liquid media (Fig 10).

4.2.2.5. Impact of *Azospirillum* population on nitrogenase activity

The population level of *Azospirillum* in the soil greatly influenced the acetylene reduction activity. There was a positive correlation between the population level and acetylene reduction up to the population level of $1\text{x } 10^8$ after which there was a reduction (Fig 11). It was also clear from the result that the population level should be between $1\text{x}10^7$ and $1 \text{ x } 10^8$ to get a significant acetylene reduction.

4.2.2.6. Nitrogenase activity in soil

The nitrogenase activity in soil samples inoculated with *Azospirillum* from their respective region was estimated *in vitro* condition. The result showed that the activity was higher in Anamallais and Vandiperiyar soil (Fig 12).

When *Azospirillum* strains from different agroclimatic zones were mixed with Anamallais soil, high nitrogenase activity was noticed with Anamallais (AN 45) and Vandiperiyar (CT 8) strains followed by M 2 (High Range) and AB (Anamallais) (Fig 13).

4.2.2.7. Effect of *Azospirillum* inoculation on ARA of roots

The acetylene reduction activity of root samples of the plants inoculated with different strains of *Azospirillum* was estimated. *Azospirillum* inoculation significantly increased the nitrogenase activity compared to the control plants. There was a wide variation in the acetylene reduction activity between the strains. The result showed that the activity was higher with strains CT 8 (Vandiperiyar), AB (Anamallais) and M 2 (High Range) (Fig 14).

4.2.2.8. Effect of agrochemicals on nitrogenase activity

In order to study the impact of commonly used agrochemicals on nitrogenase activity, an experiment was conducted *in vitro.* The result showed that all the agrochemicals reduced the nitrogenase activity. However, Bavistin was found to be less toxic (Fig 15). Chemicals like contaf, Ethion and gramaxone retarded the nitrogenase activity to a greater extent. Glyphosate and Dicofol were not as toxic as contaf, gramaxone and Ethion.

4.2.3. Factors affecting phosphate solubilization

4.2.3.1. Impact of carbon and nitrogen sources on P solubilization

Types of carbon sources greatly affected the phosphate solubilization of PSB *in vitro*. The result revealed that glucose was the best carbon source for all the strains except GP 7, which preferred fructose. Next to glucose, fructose seems to be the best source of carbon for all the strains except NP 9. Starch was less preferred by all PSB strains. Anamallais strains (ANP 24 and PB) and MP 5 were found to be best P solubilizers (Table 28).

In vitro estimation of P solubilization with respect to nitrogen sources indicated that ammonium sulphate and ammonium nitrate are the best nitrogen sources for all the selected strains. There was not much difference in P solubilization with ammonium sulphate and ammonium nitrate. Solubilization was comparatively low with calcium nitrate and urea as the nitrogen source. Anamallais strains (ANP24 and PB) and MP5 utilized all four nitrogen sources at the maximum extent. All the N sources poorly influenced P solubilization of Wayanad strain (WP11) and Karnataka strain (KP 24) (Table 29).

4.2.3.2. Solubilization of various sources of phosphate by PSB

$Ca_3(PO_4)_2$ was the best P source for all strains. Solubilization was maximum with PB (48.2) and least with WP 11 (25.0). $FePO_4$ and $Mg_3(PO_4)_2$ were readily solubilized by almost all strains, while $AlPO_4$ was found as poor source. The Anamallais strains utilized all the P sources to the maximum extent, while Karnataka and Wayanad strains (KP 24 and WP 11) were found as mild P solubilizers (Table 30). Anamallais strains ANP 24 and PB solubilized $FePO_4$ and $Mg_3(PO_4)_2$ to the maximum extent.

4.2.4. Ecology

4.2.4.1. Population dynamics of *Azospirillum* and PSB in the rhizosphere and non rhizosphere soil

A field experiment was conducted to find out the population level of *Azospirillum* and PSB in the rhizosphere and non rhizosphere soil. The result indicated that the population level of both *Azospirillum* and PSB were higher in the rhizosphere soil compared to the non rhizosphere soil. It was also noticed that the population level of these organisms were higher in clones UPASI-7, UPASI-8 and UPASI-13. The rhizosphere effect was also higher in these clones. UPASI-2 and UPASI-3 exhibited a higher rhizosphere effect with respect to PSB (Table 31).

4.2.4.2. Population dynamics of *Azospirillum* and PSB with respect to age of the bushes

Population dynamics of *Azospirillum* and PSB in tea soil was studied in relation to the age of the bushes. In general, population of *Azospirillum* was more in young plantation, while that of PSB was higher in old plantation. In addition to this, soil analysis data revealed that a gradual increase in available as well as total N and a decrease in available P in the soil samples collected from areas planted in 1960s to 1990s (Table 32).

4.2.4.3. Effect of agrochemicals on *Azospirillum* and PSB population

To study the effect of agrochemicals on the population level of *Azospirillum* and PSB in the soil, an experiment was conducted under nursery condition. The results indicated that the application of agrochemicals significantly reduced the population level of both the organisms. Among different agrochemicals tested, soil application of urea and MOP had the highest adverse effect on *Azospirillum* and glyphosate on PSB (Table 33).

4.3. Chapter III

MASS MULTIPLICATION OF BIOINOCULANTS

The mass production technology for biofertilizers involves careful selection of the microbial strains, a low cost growth medium and a suitable carrier material for a long shelf life. Quality of bioinoculants is one of the most important factors deciding their performance. A good carrier material is one which can keep up the viability of microbes for a longer period by providing organic food base to the organisms and retaining the moisture content.

4.3.1. Standardization of age and quantity of the culture

Mass multiplication of *Azospirillum* and PSB was done in fermentor using selective media. The media used were Okon's medium for *Azospirillum* and nutrient broth for PSB. The culture conditions with regard to the pH of the medium and incubation temperature were similar for both organisms. The pH of the media was kept around 7.0 and incubation temperature at 30±2°C. The results indicated that the population was on its peak on 3rd day (Fig 16) after which it declined.

4.3.2. Quantity of culture with respect to carrier material

The quantity of culture filtrate which can be mixed with the carrier material varied with their water holding capacity. In the case of lignite 200 ml/kg was found optimum, while in the case of vermicompost, organic manure 250 ml/kg and vermiculite and coir pith even with 500ml/kg, did not become pasty (Table 34).

4.3.3. Preparation of bioformulation

Three day old culture was mixed with the carrier material to standardize the volume required. As the quantity of liquid culture increased, the population was also increased gradually. Addition of 200 ml liquid culture was found as optimum for lignite, above which the formulation became paste like. In the case coir pith, addition of liquid culture did not pose such problem due to its high water holding capacity. Population enumeration showed that there was not much variation between the population level when 400 ml and 500 ml of liquid culture was added to 1 kg of coir pith (Fig 17).

4.3.4. Shelf life

The shelf life of bioinoculants (*Azospirillum* and PSB) in different carrier materials (composted coir pith, lignite, vermicompost, organic manure and vermiculite) was studied *in vitro* conditions. The result indicated that even after one year, the population level of bioinoculants was higher in composted coir pith followed by vermicompost for both *Azospirillum* and PSB. The maximum proliferation was occurred in composted coir pith followed by vermicompost and vermiculite for both *Azospirillium* and PSB strains. The proliferation was low in organic manure and lignite (Table 35 and 36).

4.4. Chapter IV

NURSERY AND FIELD PERFORMANCE OF TEA PLANTS TO BIOFERTILIZER APPLICATION

4.4.1. Effect of *Azospirillum* on rooting of tea clone

Application of bioformulations in the rooting zone of the sleeves adversely affected the rooting of cuttings (Table 37). Incorporation of bioformulations increased the pH as well as EC of the nursery soil. The cuttings exhibited club callusing in these treatments (Plate 11). Among the three clones tested, rooting was low in UPASI-3, while the other two clones UPASI-9 and UPASI-26 exhibited 90 - 100 per cent rooting.

Attempts were made to standardize the zone of biofertilizer application in the nursery. The results indicated that the response to inoculation was more prominent when the application was done in the growing zone than in the rooting zone. Addition of composted coir pith alone also increased the biometric parameters of the nursery plants, but it was significantly lower compared to that with bioformulation. This trend was same in both seedling and VP cutting plants (Table 38).

Since the application of bioformulation in the rooting zone of the sleeves adversely affected the rooting of cuttings, attempts were made to standardize the time of application of biofertilizers in the nursery sleeves. Application of *Azospirillum* formulation at different time interval indicated that the response to inoculation was more prominent when the application was done two months after putting out cuttings (C) (Table 39). In sub treatment C, the coir pith formulation increased the plant height and seedling strength than other formulations.

In sub treatment A and B, the liquid culture gave better result compared to other formulations (Table 39).

4.4.2. Colonization of *Azospirillum* and PSB on root system

The root system of nursery plants (seedlings and clones) was cut into three parts by cutting the sleeves at 10 cm length (Top, middle and bottom zones). The root samples were separated and subjected to the enumeration of *Azospirillum* and PSB in rhizosphere and non-rhizosphere soil. The result indicated that the population level was higher in the bottom zone (growing zone/zone of elongation) of the root system followed by middle zone. The population level was higher in rhizosphere region compared to non rhizosphere region. The trend remained the same in both seedlings and clonal plants (Table 40).

4.4.3. Dual inoculation of *Azospirillum* and PSB on N and P-metabolism in soil

The effect of dual inoculation of *Azospirillum* and PSB on N-metabolism and P-metabolism in nursery plants and soils was tested. The experiment revealed that the dual inoculation of *Azospirillum* and PSB improved the nitrogen and P metabolism and production of growth hormones compared to control. In *Azospirillum* treatments an increase in total N and in PSB treatments increase in available P and phosphatase activity was noticed irrespective of the carrier material. However, an increase in these properties was higher in coir pith based bioinoculant treatment. In addition to these, the inoculation showed an increase in IAA and GA3 in soil and population build up of the respective organisms. The population build up was higher in coir pith based bioformulation compared to lignite based bioformulation (Table 41).

4.4.4. Performance of *Azospirillum* strains isolated from various agroclimatic zones

To find out the nursery performance of various agroclimatic strains, an experiment was conducted. Incorporation of *Azospirillum* increased shoot (Plate 12A) and root growth (Plate 12B) and dry weight of both VP plants and seedlings. In both VP and seedlings AB, M 2 and AN 45 were superior in promoting the growth (Table 42). The response to inoculation was higher in seedlings than VP plants especially to that on root development.

The effect of various strains of *Azospirillum* on nitrogen metabolism was also tested. The total N content in soil and NR activity in the leaf were increased in *Azospirillum* treatments compared to that of control. Total soil N was higher in G 16 and C 26 treatment with clonal tea, while NR activity was higher with CT 8. In addition to these, the plant growth substance such as IAA and GA3 were also increased in soil due to *Azospirillum* treatment. IAA was more in soil treated with G 16, while GA3 was higher in C 26 treated soil.

In seedling tea, the trend was little different. Total N was higher in soil treated with AB and AN 45 strains; the NR activity was higher in plant treated with AN 45 and C 26. IAA was higher in soil treated with strains AB and M 2 while GA3 was higher in C 26 and AN 45 (Table 43).

4.4.5. Effect of *Azospirillum* and PSB on field grown plants

4.4.5.1. Response of various tea clones to bioinoculants

An experiment was conducted to study the response of various tea clones to bioinoculants. The experiment was conducted with 13 UPASI clones in the germplasm collection. The result indicated that the yield was higher in treated plots with most of the clones. The response varied with clones. The yield response was higher with clones UPASI-7 followed by UPASI-9 and UPASI-13 (Table 44). These clones showed natural preference of *Azospirillum* and PSB. The population level of *Azospirillum* was higher with UPASI-7 followed by UPASI-8, while that of PSB was higher in UPASI-17 followed by UPASI-14 and UPASI-11 (Table 44a).

4.4.5.2. Evaluation of carrier materials

The native strains of *Azospirillum* and PSB were mass multiplied and mixed with various carrier materials and applied to the field. There was a variation in the response of seedling and clones with respect to the carrier material. The yield response was higher with coir pith formulation in seedling tea (4044 kg/ha) followed by vermiculite (3968 kg/ha). The population level of *Azospirillum* was higher in vermiculite followed by coir pith. In the case of PSB it was on lignite treatment followed by coir pith.

In the case of clonal tea, vermiculite gave best response (4770 kg/ha) followed by coir pith (4533 kg/ha). With regard to population level, *Azospirillum* was higher in vermiculite and coir pith and PSB was higher in lignite and organic manure (Table 45).

4.4.5.3. Optimization of dosage of biofertilizers in tea

The result indicated that 25 kg/ha was required to bring out sustainable production and 12.5 kg/ha was insufficient. There was no significant difference in yield between 25 kg and 50 kg/ha. Biofertilizers @ 25 kg/ha with 75 % nitrogenous fertilizers and 50 % phosphatic fertilizer gave better yield response than biofertilizers @ 12.5, 20 and 50 kg/ha. This yield response was comparable with standard chemical treatment. By applying biofertilizers @ 25 kg/ha, there was a saving of 25 % nitrogenous fertilizers and 50 % of phosphatic fertilizers (Table 46; Plate 13A).

The population build up of *Azospirillum* was higher in treatment with 25 kg/ha and PSB was higher in 50 kg/ha followed by 25 kg/ha. The soil nutrient status was higher in these treatments or on par with that of standard treatment. Soil total N and available P were higher in treatment with biofertilizer @ 25.0 kg/ha, 75 % of nitrogenous fertilizer and 50 % of phosphatic fertilizer (Table 46a).

4.4.5.4. Standardization of method of application of biofertilizers

A field experiment was conducted to standardize the method of biofertilizer with seedling (Jessie) tea (Plate 13B). The biofertilizers were applied by two methods *ie.* placement and broadcasting. The yield was higher in biofertilizers applied by placement than the broadcasting method. The maximum yield was obtained in the treatment with placement of biofertilizers along with 60 % of nitrogenous fertilizer and 50 % of phosphatic fertilizer. The yield was comparable with standard treatment (Table 47). Method of application of biofertilizers was reflected in soil nutrient status as well as population build up. Total N and available P were higher in plots where the bioinoculants were applied as placement compared to broadcasting. Placement of *Azospirillum* increased soil N content and PSB increased soil available P in all treatments. The population level of *Azospirillum* and PSB were also higher in placement compared to broadcasting (Table 47a).

4.4.5.5. Frequency of application of biofertilizers in tea

A field experiment was conducted to find out whether the biofertilizers can apply once in a year or as split application. The result indicated that the split application (vide 25.0 kg/ha/year in two splits of 12.5 kg each) was superior to one time application (25 kg/ha/year in single split) with respect to yield, soil nutrient level and population build up. Application of 80 % nitrogenous fertilizer and 50 % phosphatic fertilizer with split application of biofertilizer gave better yield than single application (Table 48). The soil N and available P were higher in split application than one time application. The population builds up of *Azospirillum* and PSB were higher in split application compared to single time application (Table 48a).

4.4.5.6. Effect of biofertilizers on high yielding and low yielding tea fields

An experiment was carried out to study the response of low and high yielding field to biofertilizer application with the clone SA 6. The result indicated that biofertilizer application increased the yield in both high and low yielding fields. However, the yield response was higher in low yielding tea especially with regional strains. In the case of high yielding field, there was not much variation between regional and common strains. Nitrogenous fertilizers

@ 80 % and 50 % phosphatic fertilizers with biofertilizers gave better yield comparable to the standard treatment (Table 49).

4.4.5.7. Evaluation of biofertilizers in clonal tea

The response of clonal tea to biofertilizers was tested in a field experiment with the popular clone UPASI-9. The result showed that nitrogenous fertilizer @ 80 % and phosphatic fertilizer @ 50 % with *Azospirillum* and PSB gave yield comparable to that of standard (100 % nitrogenous fertilizer and phosphatic fertilizer). The result also indicated that the reduction in nitrogenous fertilizer up to 40 % and P fertilizer up to 50 % along with respective biofertilizers did not reduce the yield. The impact of combined inoculation of *Azospirillum* and PSB was more compared to their effect when given alone (Table 50). The application of biofertilizer increased the population level in the respective organisms in the rhizosphere. Population level of *Azospirillum* was higher in treatment of biofertilizers with 80 % nitrogenous fertilizer and 50 % phosphatic fertilizer (Table 50a).

4.4.5.8. Multilocational trials of biofertilizers

Multilocational trails were conducted in High Range (Kerala) and Nilgiri -Wayanad (TamilNadu) with seedling and clonal (UPASI-9) tea respectively. In the experiment, native strains as well as common strains (M 2 and MP 5) were evaluated.

In both cases (High Range and Nilgiri - Wayanad) standard treatment exhibited highest yield. In treatments where *Azospirillum* and PSB were applied with a reduction in nitrogenous and phosphatic fertilizers, the yield has come down. However, the incorporation of biofertilizers minimized the yield loss. There was a clear difference in the response of seedling and clone to biofertilizer application. In seedling, treatment in which nitrogenous fertilizer was reduced by 40 % and phosphatic fertilizer by 50 % along with biofertilizers yielded maximum, where as in clonal tea 20 % reduction of nitrogenous fertilizer and 50 % reduction of phosphatic fertilizer offered maximum yield. With respect to yield response, the regional strains were superior in Nilgiri - Wayanad where as in High Range the common strains were superior (Tables 51 and 52).

The population build up of *Azospirillum* was higher in High Range and PSB in Nilgiri - Wayanad. In High Range, the population build of common strains was higher than regional strains where as in Nilgiri - Wayanad, the regional strains survived better than common strains (Tables 51a and 52a).

4.5. Chapter V

ROLE OF BIOFERTILIZERS IN ORGANIC FARMING

Excessive use of inorganic fertilizers over the years reduced soil fertility. Hence a widespread need has arisen to go in for organic farming. The efficiency of sole organic inputs in nutrient management was studied through the use of different types of organic manures. Organic farming is a productive system, which reduces or avoids entirely the use of chemical fertilizers and pesticides, growth regulators and other agricultural chemicals. The system relies on crop rotation, organic manure and biofertilizers for nutrient supply, biopesticides and biocontrol for pest and disease control and innovative crop husbandry practices for maintaining soil productivity.

An experiment was conducted to study the impact on the usage of organic and biofertilizers on tea production. The standard treatment gave the highest yield. Application of organic inputs like compost, neem cake, wood ash and rock phosphate along with biofertilizers provided yield comparable to standard. However, in treatments where organic inputs were given without biofertilizers there was a significant reduction in yield (Table 53).

The total N as well as available P was higher in organic treatment compared to standard. There was a tremendous increase in the population level of *Azospirillum* and PSB in treatments where their incorporation was supplemented with organic manure (Table 53a).

DISCUSSION

In tea soil, the population level of *Azospirillum* was higher than PSB. The population level of these organisms was influenced by age of the tea bushes, nature of the soil and cultural practices. The soil temperature, moisture level, pH, atmospheric temperature and rainfall influenced the soil microflora (Pandey and Palni, 1999). Tea roots secrete root exudates that contained antimicrobial metabolites which also influenced the quantity and quality of rhizosphere microflora (Pandey and Palni, 1996). Plantation crops like arecanut, cashew, cocoa, rubber, cardamom and sapota grown in acidic soils colonized *Azospirillum* in their root system (Govindan and Purushothaman, 1985; Hu *et al.*, 2006). Gajendiran and Mahadevan (1989) isolated nitrogen fixing bacteria from tree species of *Tectona grandis, Mangifera indica, Helicterus isora, Themeda cymbaria* and *Anogeissus.* Nair and Subba Rao (1977) found that mixed cropping system stimulated the population of nitrogen fixing bacteria in the rhizosphere of coconut. Population level of PSB also varied with soil type, climatic conditions and cropping history (Kucey, 1983 and Gupta *et al.*, 1986). Yadav and Singh (1991) opined that the large variation in the population of *Azospirillum* and PSB in different soils was due to the difference in organic carbon content and types of crops (Gaind, 1987).

The population level of both *Azospirillum* and PSB were higher in the rhizosphere soil compared to the non rhizosphere soil and also there was a clonal preference. Increase in microbial population in the rhizosphere zone was attributed to the influence of factors like root exudates and decaying root materials. Rhizosphere effect indicated the affinity of microorganism to its host plant. Johri *et al*. (1999) found greater number of PSM in rhizoplane and rhizosphere than root free area of *Prosopis juliflora* and in rhizosphere of wheat, maize, cow pea, groundnut, soyabean, green gram, and rye grass (Yadav and Dadarwal, 1997). Rhizosphere is the zone of increased microbial activity immediately around the roots of higher plants (Harmastini *et al.,* 1990)

Among ten *Azospirillum* strains, eight were identified as *Azospirillum lipoferum* and the remaining two as *A. brasilense*. Among PSB strains, seven were identified as *Pseudomonas putida,* two as *P. fluorescens* and remaining one as *Bacillus megaterium*. This indicated that *A. lipoferum* and *Pseudomonas* spp. were dominant species/genera in tea soils. Isolates from jackfruit, breadfruit, sweet potato, turmeric were identified as *A. lipoferum* and that from coconut, cocoyam, elephant foot yam and sugarcane as *A. brasilense* (Govindan and Purushothaman, 1985) .

RAPD profile of selected *Azospirillum* and PSB strains was studied with random primers and developed dendrogram. Diversity within certain species of nitrogen fixing soil bacteria including *Azospirillum* (Fani *et al.,* 1993) and *Bacillus* (Mavingui *et al.,* 1992) has already been reported. The dendrogram constructed based on the dissimilarity coefficient showed grouping of the isolates into different clusters based on the existence and occurrence. The dendrogram of different isolates of *Pseudomonas* was constructed using RAPD markers showed 60 per cent dissimilarity among *Pseudomonas* associated with pearl millet, cotton and paddy (Loganathan *et al.,* 1999). It was well known that organic soil had higher diversity than sandy soil due to their physico-chemical complexity (Torsvik *et al.,* 1996).

Biology of *Azospirillum* and PSB

Tolerance to pH and temperature, resistance to antibiotics, fungicides, and pesticides, utilization of carbon, nitrogen and amino acid sources varied within the selected strains of *Azospirillum* and PSB. These variations are mainly due to the nature of strains and differences in the species of respective genus. The pH ranges for the optimum growth of *A. amazonense, A. lipoferum* and *A. brasilense* strains isolated from variety of habitats were found to be 5.7 - 6.5, 5.7 - 6.8 and 6.0 - 7.3 respectively (Baldani *et al.,* 1986b). Acidic pH was favourable for the growth as well as solubilization of P by PSB (Sperber, 1958a; Molla *et al.,* 1984). In the present study it was found that the preferential temperature for the growth of *Azospirillum* and PSB strains was 15 to 35ºC above and below which the growth was retarded. The optimum temperature for growth of *Azospirillum* was reported to be between 32 to 40ºC (Day, 1977), while PSB preferred a temperature range of 30 - 35°C (Gaind and Gaur, 1999).

Similarly, a variation was noticed among the selected strains on their tolerance level to various antibiotics, pesticides and fungicides. Similar observations were made by a number of workers (Gadkari, 1987; Bezbaruah, 1999; Rai, 1986). Balandreau (1986) observed variation in the resistance level of microorganism belonging to same species isolated from comparable habitats or even from the same habitat. Dobereiner and Baldani (1979) reported that *Azospirillum lipoferum* and *A. brasilense* showed low level of resistance to streptomycin. Mishra *et al*. (1979) reported that *S. lipoferum* was able to tolerate up to 500 ppm of streptomycin, 100 ppm of novobiocin and 10 ppm of kanamycin. *Azospirillum amazonense* strains were resistant to penicillin but relatively tolerant to chloramphenicol and erythromycin (Magalhaes *et al.*, 1983). Govindarajan and Prushothaman (1984) screened 80 isolates of *Azospirillum* with respect to tolerance of antibiotics and found that almost all isolates had low level of tolerance to streptomycin.

Agrochemicals especially pesticides and herbicides had adverse effect on *Azospirillum* growth (Gadkari, 1987) and incorporation of insecticides in the growth media caused either cell disruption or formation of cyst-like bacteria. The pesticides generally reduced the population counts and inhibitory effect varied with different pesticides in tea soils (Bezbaruah, 1999). On the other hand, application of herbicides in very low concentration significantly augmented the proliferation of aerobic non- symbiotic nitrogen fixing bacteria in the rhizosphere but in higher concentration the effect was negative (Debnath *et. al.*, 2002). They pointed out that the microorganisms utilized the herbicides and their degraded products for their growth and development.

There was a marked difference in the carbohydrate utilization between the species of *Azospirillum. A. lipoferum* grew on D-glucose but *A. brasilense* did not (Goebel and Krieg, 1984; Loh *et al.*, 1984; Martinez- Drets *et al.*, 1984). Konde (1984) reported that *Azospirillum brasilense* grew well in succinate, glycerol, D-fructose, D-gluconate, D-galactose, and L-arabinose but not on D-glucose, while *Azospirillum lipoferum* grew well in succinate, glucose, and mannose and α-ketoglutaric acid. *Pseudomonas fluorescens* utilized variety of carbon compounds as energy source. Among the carbon sources tested, glucose was found to be best followed by galactose for both tricalcium phosphate and rock phosphate solubilization (Dave and Patel, 2003).

The nitrogen sources were poorly utilized by *Azospirillum* strains, while nitrogen compounds supported the growth of PSB strains. It was observed that *Azospirillum* isolates fairly utilized both inorganic and organic nitrogenous compounds (Konde, 1984). *A. brasilense* preferred organic acids such as malate, succinate, lactate and pyruvate, (Burris *et al.*, 1978; Dobereiner, 1983a).

Dave and Patel (2003) experimentally proved that among various N sources tested, ammonium sulphate and ammonium nitrate were best for the solubilization of rock phosphate and tricalcium phosphate and urea and asparagine were the least for *Pseudomonas fluorescens.* Sodium nitrate, calcium nitrate and potassium nitrate were moderate sources of N for P solubilization. In contrary, the findings of Gaur and Sachar, (1980) showed asparagine as the best nitrogen source for P solubilization by three strains of *Pseudomonas striata.*

All the amino acids supported the growth of *Azospirillum* and PSB strains. The preference of amino acids differed from strains to strains for both *Azospirillum* and PSB. Amino acids such as L-cystine and L-tyrosine supported good growth of both *A. brasilense* and *A. lipoferum* (Konde, 1984).

There was a variation in the ammonia excretion by *Azospirillum* strains. The time required for the maximum production of ammonium was 12th day and then declined. It was found that three promising strains of *Azospirillum* were able to release ammonia in the culture medium which was influenced by the incubation period (Kundu, 1987). He suggested that this may be due to cell lysis or impairment in assimilation. Hartman *et al.* (1983) reported that during early stages of growth, these organisms utilized the fixed ammonia present in the vicinity for their own growth but subsequently released into the medium. Excretion of ammonia is a desirable character of diazotrophic bacteria which contribute towards plant productivity (Anand *et al.*, 1999).

Kundu (1987) observed that ammonia excretion took place only after the exhaustion of carbon sources. Variation in medium composition showed remarkable effect on release of ammonia. He concluded that *Azospirillum* under limited carbon availability released a part of their fixed nitrogen as ammonia and it was varied with strains to strains.

Poly -β- hydroxy butyrate is a common reserve food material in the bacterial world which imparts tolerance to higher temperature (Tal and Okon, 1985). Present study showed the existence of wide variation in the production of PHB by different *Azospirillum* strains. Similar observation was reported by Purushothaman and Vijila (1989). They have observed the accumulation of PHB in the *Azospirillum* cells that grown at 50ºC confirming its involvement in imparting temperature tolerance.

A positive correlation was observed between amount of EPS production and *in vitro* N fixation. The strains M 2 and AN 45 produced higher amount of EPS and possessed higher N fixation. Similar observation was made on *Azospirillum* isolated from *Pennisetum americanum* L. (Garg *et al.,* 1996) and opined that *Azospirillum* produced higher concentration of EPS and helped in the colonization of host roots. This view was supported by Michiels *et al.* (1989) and they have observed that the anchoring of *Azospirillum* on root surface was increased by the production of EPS. Wani *et al.* (1976) reported that the EPS production was higher in stationary culture compared to shake culture and production was positively correlated with population level of particular strain. A very strong correlation was observed between EPS production and nitrogen fixation by N-fixers.

PSB strains produced more EPS compared to *Azospirillum*. Among PSB, it was maximum with MP 5 (*Pseudomonas putida*) and least in WP 11 (*Bacillus megaterium*). On the contrary, Tank and Saraf (2003) reported higher EPS production in *Azospirillum* isolate compared to *Pseudomonas* and *Bacillus* isolates. It has been proved that EPS production provides several ecological advantages to the organisms (Garg *et al*., 1996).

Production of siderophore was not significantly varied between *Azospirillum* strains. Mori *et al*. (1992) observed that almost all azospiral strains produced siderophores for the conversion of nitrogen to ammonia. Production of siderophores was influenced by the environmental factors and media composition, especially carbon source and trace elements (Sharma and Johri, 2003). It was also reported that the production of siderophores positively correlated with nitrogenase activity and culture conditions (Shah *et al.,* 1993).

Among PSB strains, *Pseudomonas* spp. produced more amount of siderophore compared to *Bacillus megaterium* (WP 11). This finding strongly supported by Chincholkar (2000) that among large diversity of bacteria, pseudomonads were characterized by the production of siderophores. This report was further supported by Meyer and Abdallah (1978) and Cox and Adams (1985) that *Pseudomonas fluorescens, P. putida* and *P. aeruginosa* produced different types of siderophores. It has been experimentally proved that among other microorganisms, fluorescent pseudomonads are known to produce higher amount of siderophores (Sharma and Johri, 2003; Subba Rao, 1977) and this was attributed to their biocontrol potential.

Siderophores act as growth factors and/or antibiotics. Siderophore B10 inhibits the growth of *Erwinia carotovora* which causes the soft rot disease of potato. Siderophore pseudobactin deprived *E. carotovora* of Fe by scavenging the available element in the vicinity and thus reduced disease severity by minimizing the virulence of the pathogen. Similar response was observed with *F. oxysporum* in flax seedlings and *Gaeumannomyces graminis* in wheat infected by take-all disease (Subba Rao, 1995).

Azospirillum lipoferum M produced catechol–type siderophores under iron starved conditions that exhibited antimicrobial activity against various bacterial and fungal pathogens (Shah *et al.,* 1992). Twenty seven *Azospirillum* strains produced bacteriocins that inhibited the growth of several pathogenic bacteria (Tapia-Hernandez *et al.*, 1990).

Plants inoculated with *Pseudomonas* suppressed the soil born pathogen (*Fusarium oxysporum*) and increased the seedling survivability up to 90 per cent (Subba Rao, 1995). Presence of siderophore suppressed the plant pathogens *Yersinia pestis, Pseudomonas aeruginosa, Vibrio vulnificus etc.* (Litwin *et al*., 1996). The mechanism of suppression of pathogen is that the siderophore binds to the Fe^{3+} that is available in the rhizosphere and there by effectively prevent the growth of pathogenic in that region (Suslow, 1982).

The nitrogenase activity was greatly affected by biotic and abiotic factors. Addition of carbon sources had a positive impact, while nitrogen sources had a negative impact. Inorganic nitrogen sources caused significant reduction in nitrogenase activity, while organic sources such as amino acids and protein either stimulated or did not significantly inhibited the activity (Rao and Venkateswarlu, 1982). Das and Mishra (1983) observed that acetylene reduction was best with fructose and malate than succinate, pyruvate and lactate. Further, Isolates of *A. lipoferum* exhibited a higher nitrogenase activity compared to *A. brasilense* in nitrogen free medium (Han and New, 1998).

The optimum pH range for nitrogen fixation was 4.5 - 6.5. Karthikeyan (1994) isolated acid tolerant strains of *Azospirillum* that could fix nitrogen at pH levels of 4.5 to 6.0. A decrease in the nitrogenase activity was recorded when the pH was raised to 7.8. An optimum pH of 7.1 to 7.4 was recorded by Okon *et al.* (l977a) for growth and nitrogenase activity. Charyulu and Rao (1980) found that isolates originated from low pH (< 4.0) had lower nitrogen fixation than those from soils of higher pH up to 6.6.

Azospirillum required an optimum temperature of 32 - 40ºC for nitrogen fixation (Day and Dobereiner, 1976; Jagnow, 1982), a little fluctuation with in this range would not alter nitrogen fixation (Albrecht *et al.*, 1977; Dobereiner, 1980). Temperature less than 32ºC and above 45ºC inhibited the nitrogen fixation (Neyra and Dobereiner, 1977). *Azospirillum brasilense, A. lipoferum* and *A. halopraeferens* showed optimum level of acetylene reduction activity at 25, 30 and 40ºC respectively. It was found that the temperature above 35ºC inhibited both nif gene expression and acetylene reduction activity (Tripathi and Klingmuller, 1992). In the present study, optimum temperature for nitrogenase activity of *Azospirillum* was found to be 20 - 30ºC. The organism was able to tolerate up to 40°C above which the activity was retarded drastically.

There was a positive correlation between the population level and acetylene reduction up to $1x10^8$ after which there was a reduction. The population level lower than 1×10^5 and above 1×10^8 reduced the acetylene reduction. This reduction may be due the physiological requirement of the organism. This indicated the minimum requirement of the population to bring out the biological properties to a significant level.

There was a wide variation among the strains in the acetylene reduction activity in the roots. Only limited information is available on the rates of AR activity on the roots of dicotyledonous plants (Neyra and Dobereiner, 1977). Acetylene-reduction activity of *Azospirillum* in association with wheat root was between 1 - 5 and 1 - 10 n mole C_2H_4 mg dry roots^{-1} h^{-1} and the activity was strictly dependent on live bacteria in roots (Han and New, 1998). *A. brasilense* was found in association with the root population at 8×10^7 cells per gram dry weight. At this population level, the acetylene-reduction activity was found at the maximum of 2.8 µ mole C_2H_4 g dry roots^{-1} h^{-1} (Okon *et al.,* 1983).

The nature of soil greatly affected the nitrogenase activity. The activity was higher in Anamallais and Vandiperiyar soil with their respective strains. Biological nitrogen fixation in a plant-soil system was strictly dependent on the soil O_2 tension and the concentration of mineral N (Alexander *et al.,* 1987). Charyulu and Rao (1980) reported that nitrogen fixation by *Azospirillum* widely varied with soil type, soil organic matter and N content. It was noticeable that the strains with the lowest nitrogen fixation were associated with light textured and low pH soil.

Organic matter (OM) is a natural substrate for saprophytic microorganisms and provides nutrition to plants indirectly through the activity of soil microorganisms. In soil rich in organic matter, the nitrogenase activity was higher compared to those with low OM content (Subba Rao, 1995).This finding was further supported by Van Berkum and Bohlool (1980) that nitrogenase activity of *Azospirillum* was greatly affected by the difference in organic matter content. It has also been reported that the higher organic matter content of the soil increased the activity of beneficial microorganisms (Iswaran and Chhonkar, 1971).

The carbon and nitrogen sources greatly affected the phosphate solubilization of PSB. P solubilization differed between strains. This may be due to the nature of organic acids and enzymes produced by strains. Based on the nature and quantity of organic acid produced by PSB, the P solubilization differed between the strains. Glucose was the best carbon source for almost all strains. Likewise, ammonium sulphate and ammonium nitrate were the best nitrogen sources for these organisms. The form of carbon sources greatly affected the phosphate solubilization *in vitro* and *in situ* and was more active in presence of hexoses and pentoses or disaccharides (Patil *et al.*, 2001). PSM produced significant quantity of organic acids as metabolic by-products depending on various carbon sources (Bhattacharyya and Jain, 2000). Dave and Patel (2003) found that all the inorganic nitrogenous compounds supported phosphate solubilization more efficiently than the organic compounds.

The nature of phosphate sources affected the solubilization by PSB strains. Among them, $Ca_3(PO_4)_2$ was the best phosphate source for all PSB strains followed by $FePO_4$, $Mg_3(PO_4)_2$ and $AlPO_4$. Among various phosphate sources, solubilization was comparatively low with aluminium phosphate ($AlPO_4$) than other phosphate sources. The solubilization of different types of insoluble phosphate was varied with the type of microorganisms, the type of phosphates available, media conditions and available carbon sources (Yadav and Dadarwal, 1997; Kucey, 1988b). Similar results were obtained by Dave and Patel (2003) where PSB solubilized different types of P sources in the liquid medium. However, most of the strains did not show a higher P solubilization with $AlPO_4$ and $FeSO_4$ compared to Ca_3PO_4.

Mass multiplication

In fermentor, the growth was rapid compared to flask culture. This may be due to the controlled pH, temperature, aeration and nutrient supply which facilitated the rapid growth of culture in a fermentor. A viable count ranged from 10^9 to 10^{10} ml^{-1} is preferred for the preparations of bioformulation (Dadarwal *et. al*., 1997). And this population was attained within 3 - 5 days in the case of fast growing organisms such as *Azospirillum*, *Pseudomonas* and *Bacillus* sp. In the case of slow growing organisms, it took about 6-7 days to reach such counts (Dadarwal *et. al*., 1997). Most of the laboratories the practice is in using logarithmic or late logarithmic phase culture with fermentation period of 30 - 72 h or 10 - 15 day old culture (Sadasivan and Neyra, 1985).

The shelf life could be improved by paying attention to the physiological stage of the cells *eg*. high content of PHB, cysts and flocculates . The cells in the form of cysts will survive better because the cysts have storage materials and growth promoting substances (Sadasivan and Neyra, 1985). In PSB, the shelf life could be increased by using the strains in the sporulating stage. The sporulating culture was mixed with carrier materials which enhanced the shelf life of the organism (Thangaraju, 1996; Anandham *et al*., 2007). The quality of bioformulations was also determined by their shelf life. The shelf life of *Azospirillum* and PSB was higher in composted coir pith followed by vermicompost (Rajannan *et al*., 1996; Ramalingam and Ranganathan, 2001). Gaind and Gaur (1990) reported that at storage temperature of 28ºC and 35ºC and the RH had little effect on shelf life of the organisms used. Moisture content of carrier material influenced shelf life of PSB (Gaur and Gaind, 1984). Shelf life of beneficial organisms was decreased in paddy straw. This may be attributed to the production of phenolic acids composition of organic material (Gaur and Pareek, 1972). *B. polymyxa,* a spore former, could not withstand the high temperature and low moisture status of the carrier which may be due to its ability to produce polysaccharide extracellularly (Rolz and Leon, 1988).

Based on the *in vitro* and *in vivo* experiments, Muthukumarasamy *et al*. (1996) suggested that vermicompost can be used as an alternative carrier material to lignite. In addition to these, composed coir pith is a good carrier for bacterial as well as fungal inoculants (Baby, 2004). Finely ground vermiculite supplemented with nutrient sources and moisture could be used for the preparation of bacterial inoculants. An inoculant was formulated by Fages (1992) that consisted of dried micro-encapsulated bacterial cells dehydrated in a polymer matrix. In this formulation, the number of viable cells decreased to 10^9 cfu g^{-1} from 10^{12} within 90days of storage.

Present study revealed that composted coir pith is a good carrier for bacterial strains. There was a variation in the response of seedling and clones with respect to the carrier material. The quality of bioformulation is mainly depending on the nature of carrier materials. Among different carrier materials the population level of bioinoculants was higher in composted coir pith followed by vermicompost and the proliferation was lesser in organic manure and lignite. Composted coir pith kept the viability of microbes for a longer period by providing organic food base to the organism and retaining the moisture content. Dadarwal *et al*. (1997) reported that characteristics of a good carrier material are water holding capacity, pH, particle size, sticking ability on seeds and total surface area provided for absorption of microbial cells per unit weight. The composted coir pith has all these characters and the superior performance of composted coir pith formation may be attributed to this. The formulation should contain 1x 10^7 cfu g^{-1} of carrier material in the case of bacteria and $1x10^6$ for fungi (Baby, 2004).

The nature of carrier material, shelf life and inoculum potential are important in the quality of bioinoculants. The mass production technology for biofertilizers involves careful selection of the microbial strains, a low cost growth medium and a suitable carrier material for a long shelf life. Quality of bioinoculants is one of the most important factors deciding their performance. A good carrier material is one which can keep up the viability of microbes for a longer period by providing organic food base to the organisms and retaining the moisture content.

The survival of *Azospirillum* and phosphate solubilizing bacteria was better in press mud compared to peat, lignite, and composted press mud, but in the field the survival of *Azospirillum* was better in composted pressmud applied soil (Rajannan *et al*., 1996). Govindarajan (1987) studied the survival of *Azospirillum lipoferum* in peat, coir pith and peat-coir pith mixture and found that the peat supported the maximum proliferation of *Azospirillum* than other carriers. On the other hand, Sureshkumar (1996) found that pressmud was a superior carrier compared to peat and lignite. The survival of *Azospirillum* and phosphate solubilizing bacteria was better in press mud compared to peat, lignite, and composted press mud, but in the field the survival of *Azospirillum* was better in composted pressured applied soil (Rajannan *et al*., 1996).

Nursery and field performance of biofertilizers

Application of *Azospirillum* and PSB increased the plant growth and soil nutrients under both nursery and field conditions. The response varied with time, dosage, frequency and mode of application, bacterial strains and type of planting material.

Amendment of nursery soil with bioformulation increased its pH as well as EC. Among the three clones tested, rooting was poor in UPASI-3, while the other two exhibited 90 to 100 per cent rooting. In UPASI-3, the cuttings exhibited club callusing with poor root development in most of the treatments. This may be due to the increased pH and EC of the soil. UPASI-3 is known to be highly sensitive to pH and soil texture (Venkataramani and Sharma, 1969). Interference of pH on rooting success in organic manure/biofertilizer treatment in nursery has already been reported (Victor, 2000). The per cent success in other two clones could be due to their tolerance to pH. The seedlings were unaffected by the change in pH confirming their higher tolerance to pH.

The response to bioformulation was more prominent when it was incorporated in the growing zone than in the rooting zone. In addition to this, application at different time interval indicated that the response was prominent when applied two months after putting out cuttings. Tea being highly sensitive to pH, exposure of cuttings directly to higher pH and EC resulted in callusing and subsequent inhibition on the root development. In the case of treatment where in bioformulation was applied two months after putting out of cuttings had normal callusing and rooting. This further confirms the interference of pH on rooting of cuttings. Since the biofertilizer was applied at the time of root initiation, easy colonization of them on the root system was possible resulting in quick and prolonged response. Fallik *et al*. (1988) and Chakraborty *et al.* (2002) found that the time of application of *Azospirillum* influenced the root growth in maize. They observed a difference in the root surface area between pre-emergence (at the time of sowing) and post emergence (after the appearance of the second leaf) treatment.

The response to biofertilizer application was varied with different clones. This may be due to the difference in the nature of colonization, root exudates produced by plants, nature of the root system and their genetic make up. Plants and bacteria closely interact influencing their activity. Kundu *et al.* (2002) were of opinion that the genetic make up of plants determines the composition of root exudates. Michiels *et al.* (1989) suggested the chemotactic response to organic acids as one of the factors determining host - specificity in colonization.

The biofertilizer application increased the yield in both high and low yielding fields. The response to biofertilizer application was more when the soils were optimally fertilized. A significant increase in yield was achieved by the application of biofertilizers with intermediate rate of N-fertilizer (Okon and Labandera-Gonzalez, 1994; Bashan *et al.*, 2006). Application of biofertilizer can be adjusted based on the OM content of the soil, which is an integrated approach for sustainable productivity. In tea soil, OM content was positively correlated with the population level of *Azospirillum.* Baby (2002) reported that *Azospirillum* population was higher with increase in amount of OM content.

In the agricultural field, higher level of organic matter content increased the plant growth and development. High level of OM content improved the soil physical nature and it was positively correlated in the improved plant growth and yield of crop plants (Rajagopal and Ramarethinam, 1997). Higher OM content in the soil facilitated the survival of plant growth promoting organisms such as *Azospirillum, Rhizobium, Azotobacter etc.* (Tilak *et al.*, 1979). Organic matter helps to maintain high viable count of beneficial microorganisms (Tilak and Singh, 1994). High level of organic matter content increased the water holding capacity and neutral pH for better survival of the microorganisms (Date, 1968).

Organic matter influences physical, chemical and biological properties of soil despite its small proportion in the soil. It commonly accounts for at least half the cation exchange capacity of the soil and is perhaps responsible for more than any other single factor for the stability of the soil. Moreover, it supplies energy for microorganisms whose activities are of vital importance in soil dynamics (Surendra Mohan, 1995).

The response of tea plant to bioformulation was more when applied by placement compared to broadcasting. This was reflected in the yield response as well as population buildup of *Azospirillum* and PSB. In placement, the bacteria can be introduced near to the root zone facilitating their easy colonization on the feeder root. The higher response to placement may be attributed to this factor. However, the method of application should be practical, economical and easy to accomplish for the farmer. Seed dressing, placement in furrow, broadcasting are the common methods of application. All these methods provided positive results. However, the volume of inoculants needed to achieve optimal results depends on the cell concentration (Okon and Labandera-Gonzalez, 1994; Ellis, 1995; Jat and Shaktawat, 2001).

The split application of biofertilizers (two splits in a year) was superior to one time application with respect to yield, population buildup and soil nutrient status. The time of application of biofertilizers is crucial as the bacterial inoculation should be at a time when plants need it (Bashan, 1986a). The use of biofertilizers for winter wheat had limited value until obtaining an understanding on the factors influencing the rhizosphere competence of bacterial inoculants (Kucey, 1988a). Further, an optimum dosage of biofertilizer is needed to bring out the desired result. In tea, 25 kg/ha was found as optimum. In maize, an optimal density of *Azospirillum* was not required to promote yield. However, it was found that the optimal density is indispensable up to the emergence of the radicle (Jacoud *et al.*, 1998).

Application of 75 per cent of recommended dose of N:P (40:20 kg/ha) along with biofertilizers (2-4 kg/ha) significantly increased the grain and straw yield of sorghum-horsegram cropping system (Kapulnik *et al.*, 1981a; Morgenstern and Okon, 1987; Parasuraman *et al.*, 2000). In wheat, a significant increase in the yield and P uptake was observed with integrated use of rock phosphate and PSB @ 30 - 35 kg/ha (Narayanasamy *et al.*, 1981; Dubey *et al*., 1997).

The dual inoculation of *Azospirillum* and PSB increased the N-metabolism and P-metabolism in tea plants and soils under nursery condition. Dual inoculation of *Azospirillum* and PSB improved the N and P metabolism and growth hormones compared to control. In *Azospirillum* treatments an increase in total N and nitrogenase activity and in PSB treatments increase in available P and phosphatase activity were noticed irrespective of the carrier material. Dual inoculation further showed an increase in plant growth hormones in soil and population build up of the respective organisms. The positive benefits from inoculation were attributed to several mechanisms such as nitrogen fixation, phytohomones production, enhanced nutrient uptake (Wani, 1990; Barea *et al*., 1983; Kapulnik *et al*., 1981b; Fernandez *et al*., 2007) and phosphate solubilization (Frietas and Germida, 1990). Padmavathi *et al.* (1991) investigated that the dual inoculation of *Azospirillum lipoferum* and phosphate solubilizers greatly helped in the uptake of nitrogen, phosphorus, zinc, copper and iron in the shoots of foxtail millet under field conditions.

Azospirillum strains produced several plant hormones. The major hormone produced were indole -3- acetic acid (IAA) and indole -3- butyric acid (IBA) (Fallik *et al.,* 1989) and other hormones were detected at much lower, but biologically active level. These hormones include indole -3- lactic acid (Tien *et al.,* 1979), indole -3- methanol (Crozier *et al.,* 1988) and other unidentified indole compounds (Hartmann *et al.,* 1983), cytokinins (Horemans *et al.,* 1986) and several gibberellins (Bottini *et al.,* 1989). Tien *et al.* (1978) found that *A. brasilense* was able to produce gibberellins and cytokinin like substances in the liquid culture (Vlassak and Reynders, 1978). *Azospirillum brasilense* was found to produce high amounts of IAA (Horemans *et al*., 1986), however, it depended on the species and strains (Hartman *et al*., 1983; Omay *et al.,* 1993). Further, the IAA production was proportional to the bacterial population in the medium, when other factors were not limiting. Likewise, phosphate solubilizing bacteria also produced growth promoting substances such as auxins, gibberellins and cytokinins, which improved the plant growth and stimulated the microbial development (Sattar and Gaur, 1987; Sullia, 1968).

The application of bioformulation also increased the soil nutrient level such as total N and available P. In *Azospirillum* and PSB treatments, the total N and available P were increased. This increase in the soil nutrient level was responsible for plant growth and development. *Azospirillum* inoculation increased plant growth, dry weight, total nitrogen content and yield of cereal and forage grasses (Nur *et al.,* 1980; Kundu, 1988). In addition to morphological effect, there was an increase in biochemical characters (Wani *et al*., 1985). *Azospirillum* inoculated plants showed increase in the level of total nitrogen in different parts of the plant (Saxena *et al.,* 1990). Inoculation with nitrogen fixing bacteria always increased leaf NRA suggesting a greater supply of NO_3 to the plants over uninoculated control. The increased NO_3 uptake may relate to increased root development in response to production of hormones (Tien *et al.*, 1979; Tilak and Subba Rao, 1987). Ferreira *et al.* (1987) reported that wheat plants inoculated with *Azospirillum* showed greater activity of the nitrate reductase enzyme in the leaf compared to uninoculated plants.

Role of biofertilizers in organic farming

Organic farming is best suited for perennial plantation crops like tea, which provide proper shade and cover to the soil and keep the soil microbial activity constant. To keep the soil microbially active, there should be enough organic matter and hence an organic farming system will be ideal (Baby, 2003). One way of achieving organic farming is through integrated nutrient management rather than sole reliance on chemical fertilizers. For sustainable productivity, chemical fertilizers, organic manures and biofertilizers should be given as complementary to each other in a balanced way (Baby, 2002).

In the present study, application of organic inputs such as compost, neem cake, wood ash and rock phosphate along with biofertilizers gave higher yield. On the other hand, in treatment with organic manures wherein biofertilizers were not given, there was a reduction in the yield. Inoculation of biofertilizers along with organic manure gave better results than their individual application and combination of two or three bioinoculants (Rajagopal and Ramarethinam, 1997; Behera *et al*., 2007). Agronomists have visualized potential of organic farming for increase in growth and flavor quality of tea.

Organic inputs increased the soil N and P content. The population buildup of *Azospirillum* and PSB was higher in the treatments supplemented with organic manure. It was proved that application of organic manures increased the biological properties of soil that increased the populations build up of beneficial microorganisms (Subba Rao, 1995). These organic inputs are facilitating good habitats of beneficial microorganisms in the soil environment.

The activity of beneficial microorganism in soil was enhanced by the application of organic inputs. It will maintain as well as improve the population level of agriculturally important microorganisms (Calvaruso *et al*., 2007). Baby (2006) opined that the organic input as biofertilizers reduced the application of chemical fertilizers considerably in the fertilizer schedules. A reduction of 15 - 20 % of nitrogenous fertilizers and even up to 50 % of phosphatic fertilizers was possible by applying biofertilizers.

Integrated fertilizer management with finger millet (*Eleusine coracana*) revealed that seed inoculation of *Azospirillum* gave an additive effect over farmyard manure (FYM), nitrogen and phosphorus application alone and the effect of *Azospirillum* was found to be equivalent to 20 kg N/ha through urea. The combined application of *Azospirillum* and nitrogen gave better effect than *Azospirillum* with phosphorus. However, rock phosphate (RP) recorded growth and yield on par with single super phosphate (SSP). Highest dry matter, ear heads/m^2, length of finger, NPK uptake and grain and straw yield were obtained by *Azospirillum* + N_{80} followed by *Azospirillum* + N_{60} (Chakraborty *et al.*, 2002). Integrated fertilizer management with wheat increased the yield and reduced nitrogenous fertilizer requirements up to 40 kg/ha. This amount is economically important and indicated the higher potential of grass-bacteria system (Parasuraman *et al.*, 2000). Under field condition, application of organic fertilizers and biofertilizers (phosphate solubilizing bacteria and *Rhizobium*) and inorganic fertilizers (phosphorus and sulphur) gave better yield response in fenugreek (*Trigonella foenum-graecum*).

In cotton, combined inoculation of *Azospirillum, Pseudomonas striata* and 50 % urea and 100 % P as rock phosphate gave better response, nutrient uptake and population buildup of beneficial microorganisms (Prathibha *et al.*, 1994). In sugarcane, application of pressmud @ 20 kg/ha with *Azospirillum,* increased the percentage of germination of setts, length of cane and number of tillers. In rice, treatment with the inoculation of *Azospirillum* along with pressmud@12.5 kg/ha and N:P:K @ 100:50:50 kg/ha recorded maximum yield (Sundaram, 1991). The combined inoculation of bioinoculants such as *Azospirillum* and PSB with 50 % of N and P recorded higher microbial population, number of bolls/plants and kapas yield of rice fallow cotton (Thamizh Vendan *et al.,* 2000; Kundu and Gaur, 1984).

Organic matter like compost on decomposition releases humic acid (HA) which acts as a growth stimulator for plants. HA facilitates the uptake of nutrients and increase the growth performance of plants. It also enhances the microbial activity and improves soil health (Gaur *et al.*, 1971).

Organic farming is to reduce the chemical inputs and sustain crop production. Organic tea cultivation is at the conservation of ecology and natural habitat without polluting soil, air and water and yet maintaining sustainable tea production. In organic, naturally occurring, mined products and bulky and concentrated organic manures such as compost, neemcake, wood ash, bone meal, fish meal, rock phosphate, biofertilizers and biodynamic formulation are used for nutrition and maintenance of soil fertility. Organic matter influences physical, chemical and biological properties of soil despite the small proportion present in the soil.

SUMMARY AND CONCLUSION

- Totally 236 *Azospirillum* and 114 phosphate solubilizing bacterial (PSB) strains were isolated from soil samples collected from different agro climatic zones of south Indian tea plantation.

- In tea soil, the population level of *Azospirillum* was higher than phosphobacteria in all tea districts except Vandiperiyar. *Azospirillum* population was higher in Anamallais followed by Nilgiri - Wayanad and least in Karnataka. In the case of phosphobacteria, the population level was highest in Vandiperiyar and least in Wayanad.

- Based on the *in vitro* studies *Azospirillum* strains were screened for their efficiency and selected the best one/two strains from each agroclimatic zone. The ten selected strains each of *Azospirillum* and PSB were identified based on biochemical tests. Among the ten *Azospirillum* strains 8 were identified as *Azospirillum lipoferum* and remaining 2 were identified as *A. brasilense.* Among the ten PSB strains 7 were identified as *Pseudomonas putida*, 2 as *P. fluorescence* and remaining one as *Bacillus megaterium*.

- The biodiversity studies of these strains were conducted based on RAPD. The result showed that the *Azospirillum* strains AN 45, K 4, M 2, N 1 and CT 8 form the first group, G 16 and W 5 strains form the second group and AB, C 26 and M 4 form the third group. Among the PSB strains, ANP 24, PB, CP 29, KP 24, MP 17, MP 5, VP 19 and WP 11 form the first group, GP 7 and NP 9 form the second group.

- Among ten *Azospirillum* strains, M 2 and M 4 (both from High Range) were superior in fixing atmospheric nitrogen. As far as Acetylene Reduction Activity was concerned, the strain CT 8 and AN 45 were superior.

- The organic acid production was highest with strains PB and WP 11. These two strains possessed high phosphatase activity. It was also found that solubilization of phosphate from all sources was higher with strain PB.

- *Azospirillum* and PSB strains grown in fermentor attained maximum population level on 3rd day and in shake culture on 5th day. In static culture, *Azospirillum* took 6 days and PSB took 7 days to reach maximum population.

- Optimum pH level for the growth of *Azospirillum* and PSB was ranged from 5.0 to 7.5. However, *Azospirillum* strains G 16, M 4, CT 8 and AB were able to grow at pH 4.5. In the case of PSB, except for CP 29 and KP 24 all others were able to grow at pH 4.5. It was also found that the optimum range of pH for N fixation was 4.5 – 6.5.

- *Azospirillum* tolerated a range of temperature (5 - 35ºC) compared to PSB (15 - 35ºC). The optimum temperature for nitrogenase activity was 30ºC.

- The selected strains of *Azospirillum* and PSB had good tolerance to antibiotics. *Azospirillum* strains showed tolerance up to 300 ppm of penicillin, 200 ppm of chloramphenicol and 250 ppm of streptomycin. In the case of PSB, maximum tolerance level was 200 ppm for penicillin, 300 ppm for chloramphenicol and streptomycin.

- The selected strains of *Azospirillum* and PSB were able to tolerate 3000 ppm of Dicofol, 2000 ppm of Ethion, 200 ppm of COC and 500 ppm of Tilt.

- In general, agrochemicals reduced the nitrogenase activity but Bavistin was found to be less toxic. Chemicals like Contaf, Ethion and Gramaxone retarded the nitrogenase activity to a greater extent. Glyphosate and Dicofol were found as less toxic.

- Almost all the *Azospirillum* strains preferred trehalose, melibiose, xylose, rhamnose, and adonitol as carbon source. Among the organic compounds, malate served as the best carbon source followed by succinate. In the case of PSB, all the isolates except ANP 24 utilized manodione, starch, rhamnose and inositol as carbon source. Among organic compounds succinate was the best carbon source.

- *Azospirillum* strains poorly utilized the nitrogenous compounds such as ammonium sulphate, ammonium nitrate, ammonium tartrate, ammonium chloride and urea as nitrogen source. In the case of PSB, all the nitrogenous compounds supported the growth. Among them, ammonium sulphate, ammonium nitrate and potassium nitrate were found as good source.

- All the amino acids supported good growth of *Azospirillum* and PSB. Among them, tyrosine, tryptophan and methionine were preferred sources by *Azospirillum* compared to others. PSB strains preferred leucine, cystine, serine, glycine and proline. Among the strains, MP 17 utilized maximum number of amino acids and ANP 24 and CP 29 strains poorly utilized amino acid sources.

- All the *Azospirillum* strains readily utilized nicotinic acid, Vitamin B1 and folic acid. PSB strains preferred Vitamin B12, C and nicotinic acid.

- Among the ten *Azospirillum* strains, AB produced more amount of IAA, while strain G16 produced more GA3. In the case of PSB, MP 17 and MP 5 produced higher level of IAA, while PB produced more GA3.

- Among selected strains of *Azospirillum*, M 2 excreted more ammonia followed by AN 45.

- A wide variation was observed in the production of PHB. The *Azospirillum* strain M 2 produced higher level of PHB followed by CT 8 and AN 45.

- Siderophore production was more in PSB compared to *Azospirillum*. Among *Azospirillum* strains, M 2, AN 45 and AB produced higher amount of siderophore. In the case of PSB, strain PB produced more amount of siderophore followed by MP 5 and ANP 24.

- *Azospirillum* strain K 4 produced maximum EPS followed by M 2 and AN 45. Among PSB strains, EPS production was maximum with MP 5.

- The nature of culture media greatly influenced the activity of nitrogenase enzyme under *in vitro*. The semisolid medium was found to be the best for nitrogenase activity compared to solid and liquid media.

- External application of nitrogen and carbon sources greatly influenced the nitrogenase activity in the culture. Carbon sources had a positive impact while nitrogen sources had a negative impact. Among the carbon sources, malate supported the nitrogenase activity to the maximum followed by maltose. On the other hand, addition of nitrogen sources decreased the nitrogenase activity to varying level and the suppressive effect was maximum with urea followed by ammonium sulphate.

- The population level of *Azospirillum* in the soil greatly influenced the acetylene reduction activity. The population level between 1 x 10^7 and 1 x 10^8 found to be optimum for acetylene reduction activity.

- In the soil samples, the nitrogenase activity was higher when the soil samples inoculated with their native *Azospirillum* strains. Among them, Anamallais strain had higher activity than others. The result revealed that the activity was higher only in their respective soil with native stains. Same result was observed in root samples also.

- The types of available carbon sources were greatly affected the P solubilization by PSB *in vitro*. Among different types of carbon sources, glucose was the best source than fructose, sucrose and starch.

- The nature of phosphate sources greatly affected P solubilization capacity. The P solubilization was higher in the medium contained $Ca_3(PO_4)_2$ followed by $FePO_4$, $Mg_3(PO_4)_2$ and $AlPO_4$. Anamallais strains ANP 24 and PB solubilized $FePO_4$ and $Mg_3(PO_4)_2$ to the maximum extent.

- Application of agrochemicals significantly reduced the population level of *Azospirillum* and PSB. Among different agrochemicals tested, soil application of urea and MOP had the highest adverse effect on *Azospirillum* and Glyphosate on PSB.

- For mass multiplication, addition of 200 ml liquid culture was found as optimum for lignite and 400 ml for coir pith.

- The shelf life of bioinoculants was higher in composted coir pith and was lesser in organic manure and lignite.

- Incorporation of bioformulations increased the pH as well as EC of the nursery soil and resulted in the formation of club callusing and failure in rooting. Among three clones tested, rooting was low in UPASI-3, while other two clones UPASI-9 and UPASI-26 exhibited 90 - 100 per cent rooting.

- Response of tea plants to bioinoculants was more prominent when the application was done in the growing zone than in the rooting zone of nursery sleeves. This trend was same in both seedlings and VP plants. It was also found that the response was more prominent when the application was done two months after putting out cuttings.

- In tea root system, the population level was higher in the bottom zone (growing zone/zone of elongation) followed by middle zone. This trend remained the same in both seedlings and clonal tea.

- The rhizosphere effect was higher in clone UPASI-7, UPASI-8 and UPASI-13. UPASI-2 and UPASI-3 exhibited a higher rhizosphere effect with respect to PSB.

- The population level of *Azospirillum* was more in young plantation, while that of PSB was higher in old plantation. In addition to these, soil analysis data revealed that a gradual increase in available as well as total N and a decrease in available P in the soil samples collected from areas planted in 1960s to 1990s.

- Dual inoculation of *Azospirillum* and PSB increased the N-metabolism and P-metabolism in nursery plants and soils. In addition to these, the inoculation showed an increase in IAA and GA3 in the soil.
- A field study revealed that there was a clonal preference to bioinoculants. The population level of *Azospirillum* was higher with UPASI-7 followed by UPASI-8, while that of PSB was higher in UPASI-17 followed by UPASI-14 and UPASI-11.

- There was a variation in the yield response of seedling and clones with respect to the carrier material. The yield response was higher with coir pith formulation in seedling tea followed by vermiculite. In the case of clonal tea, vermiculite gave best response followed by coir pith.

- Optimum dose of biofertilizers for tea was found as 25 kg/ha for the sustainable production.

- Experiment on the standardization of method of biofertilizer application indicated that the response was more in placement than broadcasting. It was also revealed that split application was more efficient than single time application.

- Biofertilizer application increased the yield in both high and low yielding tea fields. However, the yield response was higher in low yielding tea.

- In clonal tea (UPASI-9), nitrogenous fertilizer @ 80 % and phosphatic fertilizer @ 50 % with *Azospirillum* and PSB gave yield comparable to that of standard (100 % nitrogenous fertilizer and phosphatic fertilizer).

- In multilocational field trails, the standard treatment exhibited highest yield. In treatments where *Azospirillum* and PSB were applied with a reduction in nitrogenous and phosphatic fertilizers, the yield was reduced. However, the incorporation of biofertilizers minimized the yield loss. There was a clear difference in the response of seedling and clone to biofertilizer application.

- The field experiments revealed that a reduction in 20 – 40 % nitrogenous fertilizers and 50 % of phosphatic fertilizers was possible with the addition of biofertilizers.

- Application of organic inputs like compost, neem cake, wood ash and rock phosphate along with biofertilizers provided yield comparable to standard. However, in treatments where organic inputs were given without biofertilizers there was a significant reduction in yield. The total N as well as available P was higher in organic treatment compared to standard. There was a tremendous increase in the population level of *Azospirillum* and PSB in treatments where their incorporation was supplemented with organic manure.

- In all the field experiments, the application of biofertilizers increased the population build up of *Azospirillum* and PSB. In addition to this, the soil nutrient content such as nitrogen and phosphorus were higher in biofertilizers treated soil compared to control and standard treatment.

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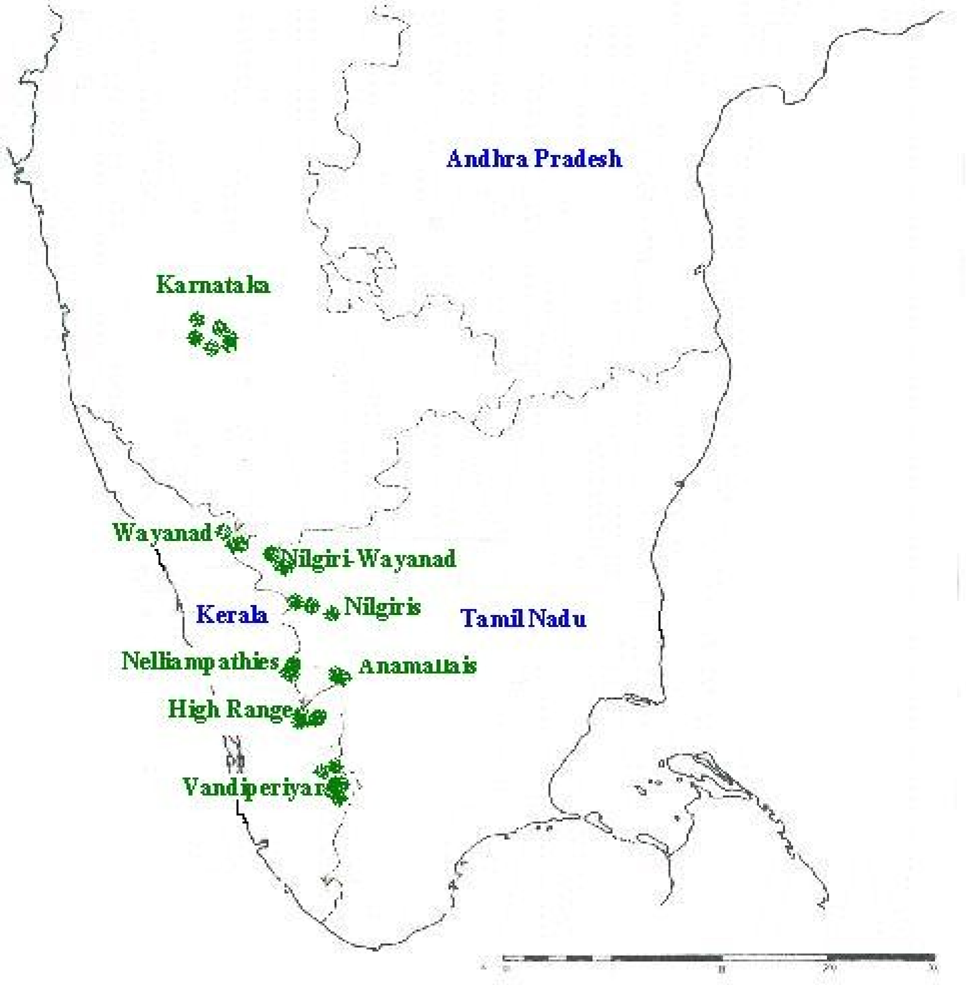


Fig 1: Collection sites of soil samples

Fig 5: Impact of carbon sources on nitrogenase activity

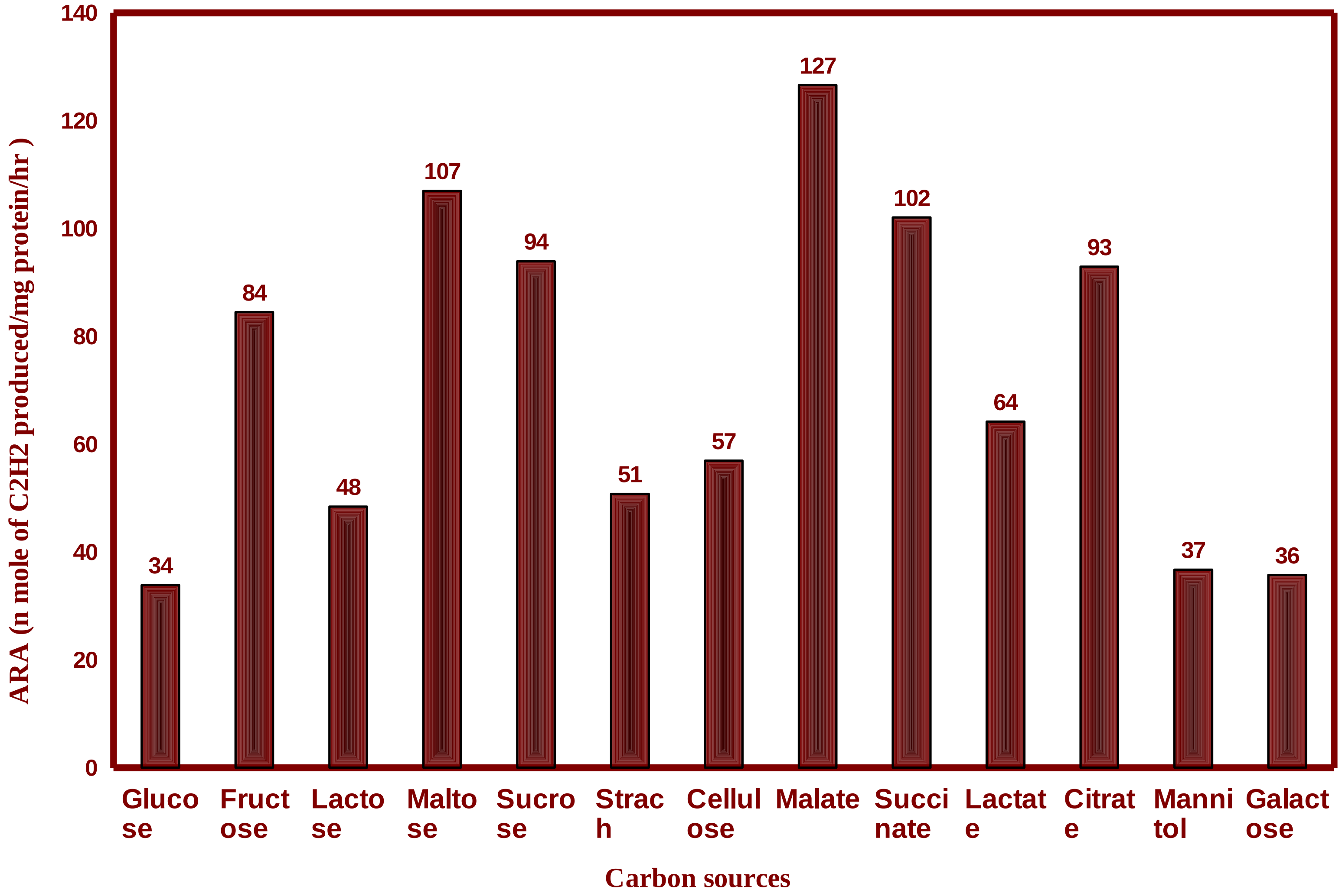
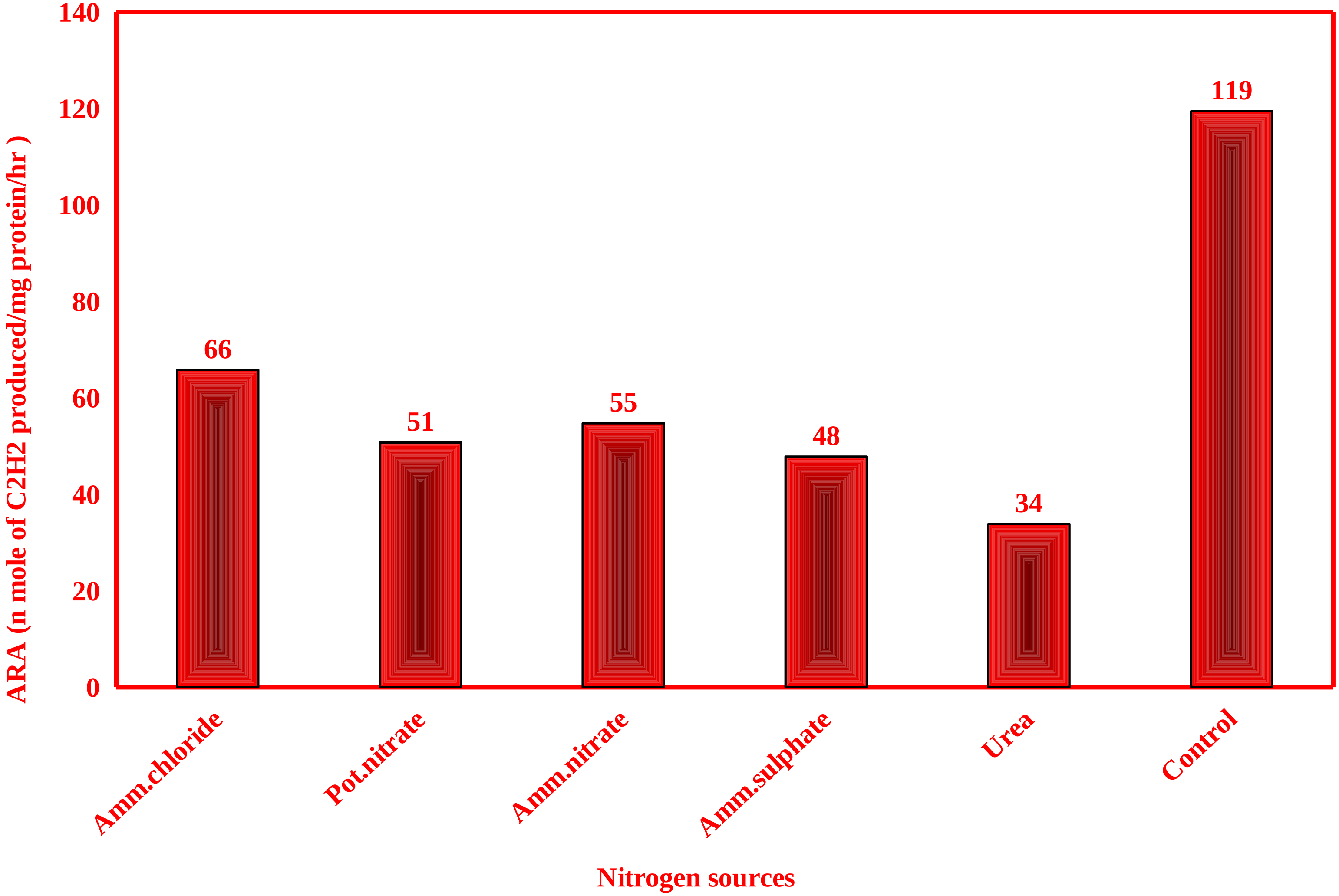


Fig 6: Effect of nitrogen sources on nitrogenase activity



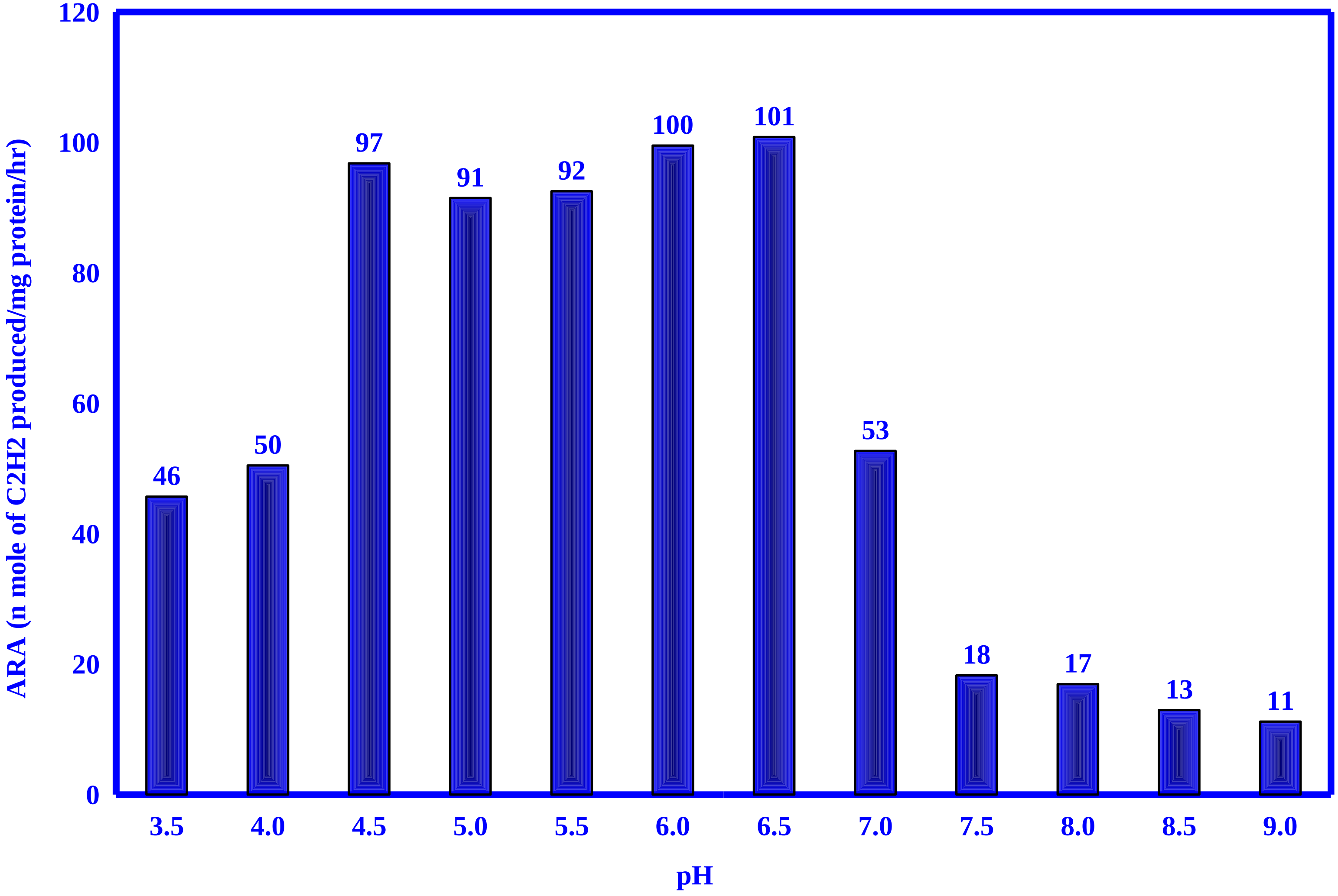


Fig 7: Effect of pH on nitrogenase activity

Fig 8: Effect of temperature on nitrogenase activity

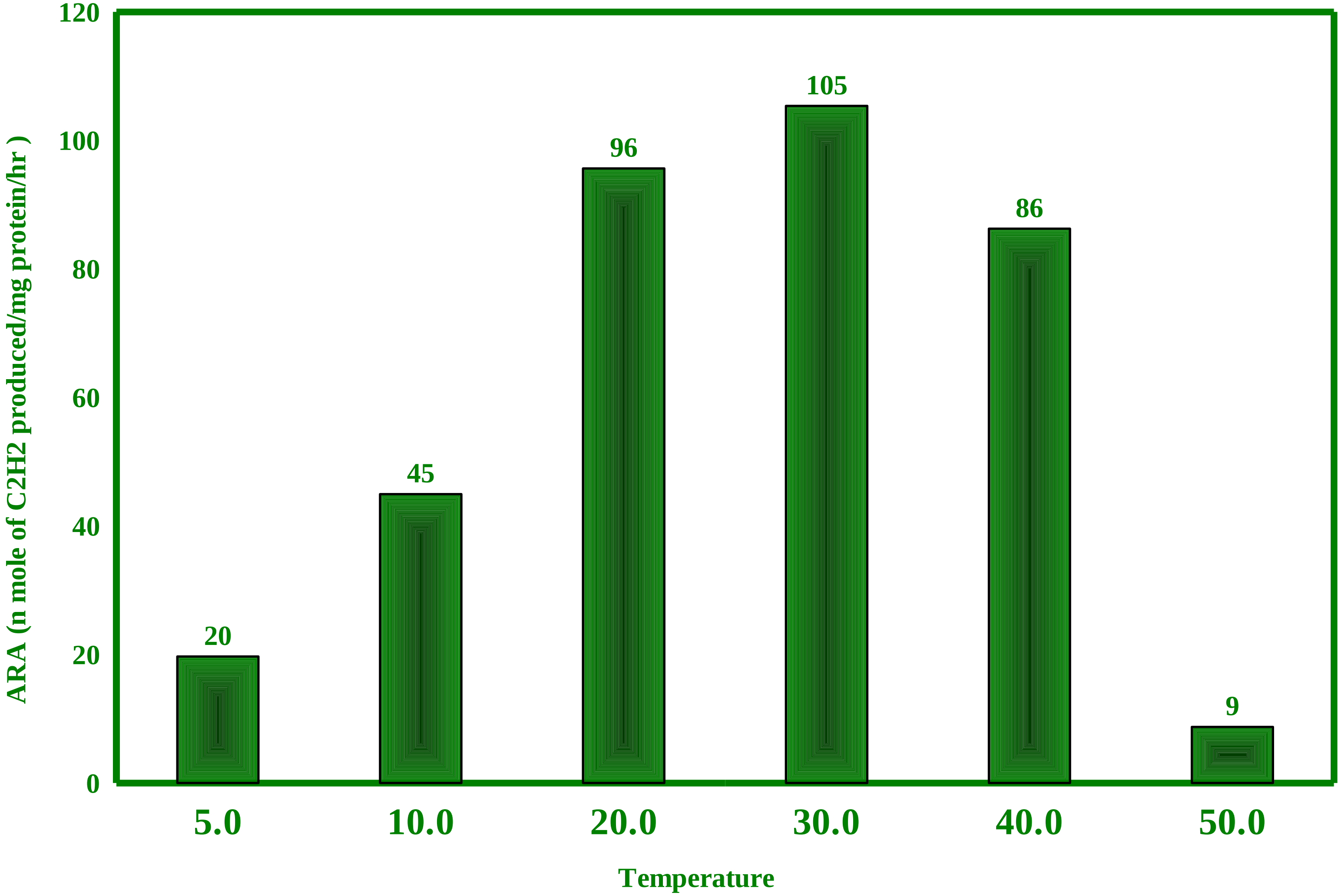


Fig 9: Effect of incubation period on nitrogenase activity

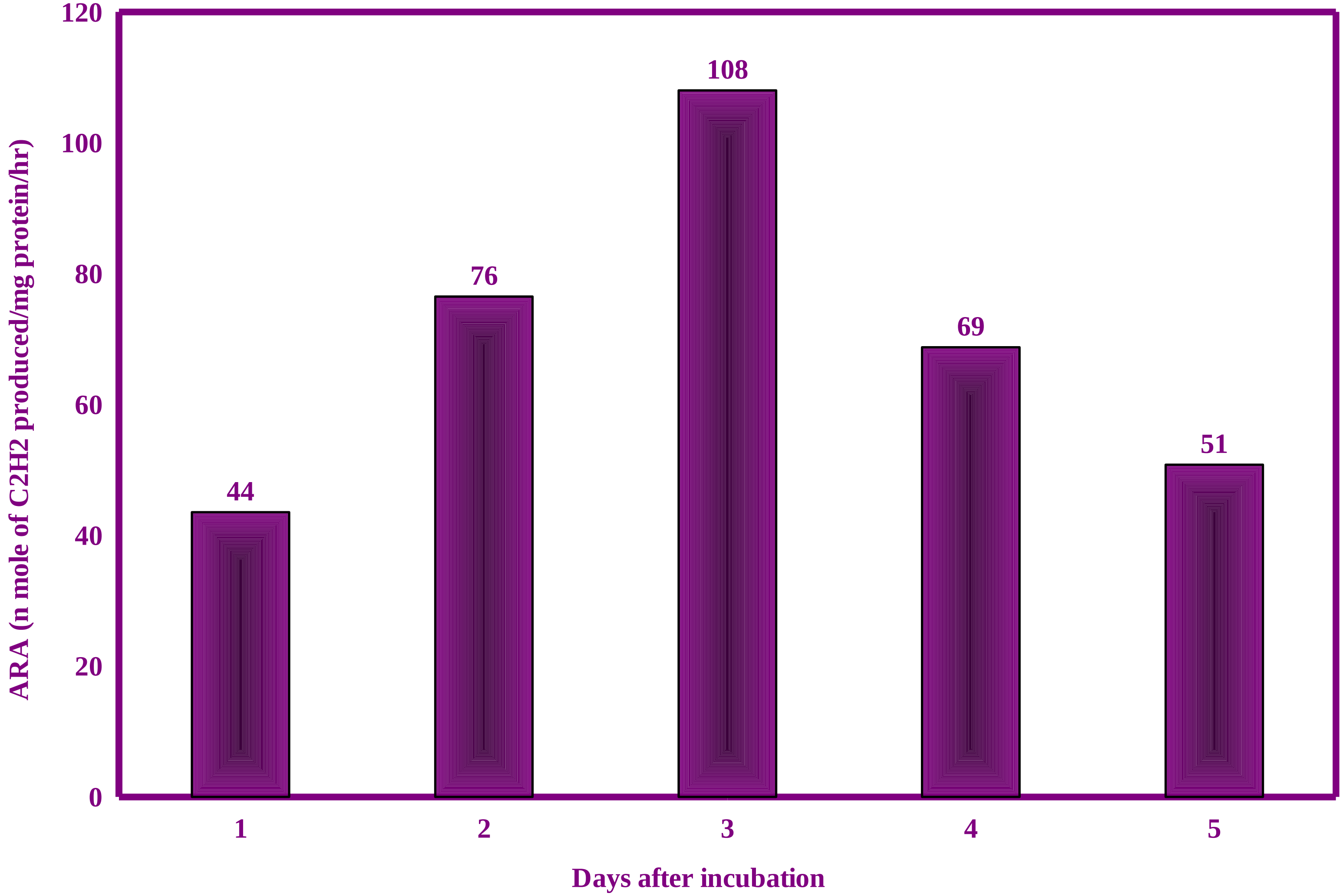


Fig 10: Effect of types of media on nitrogenase activity

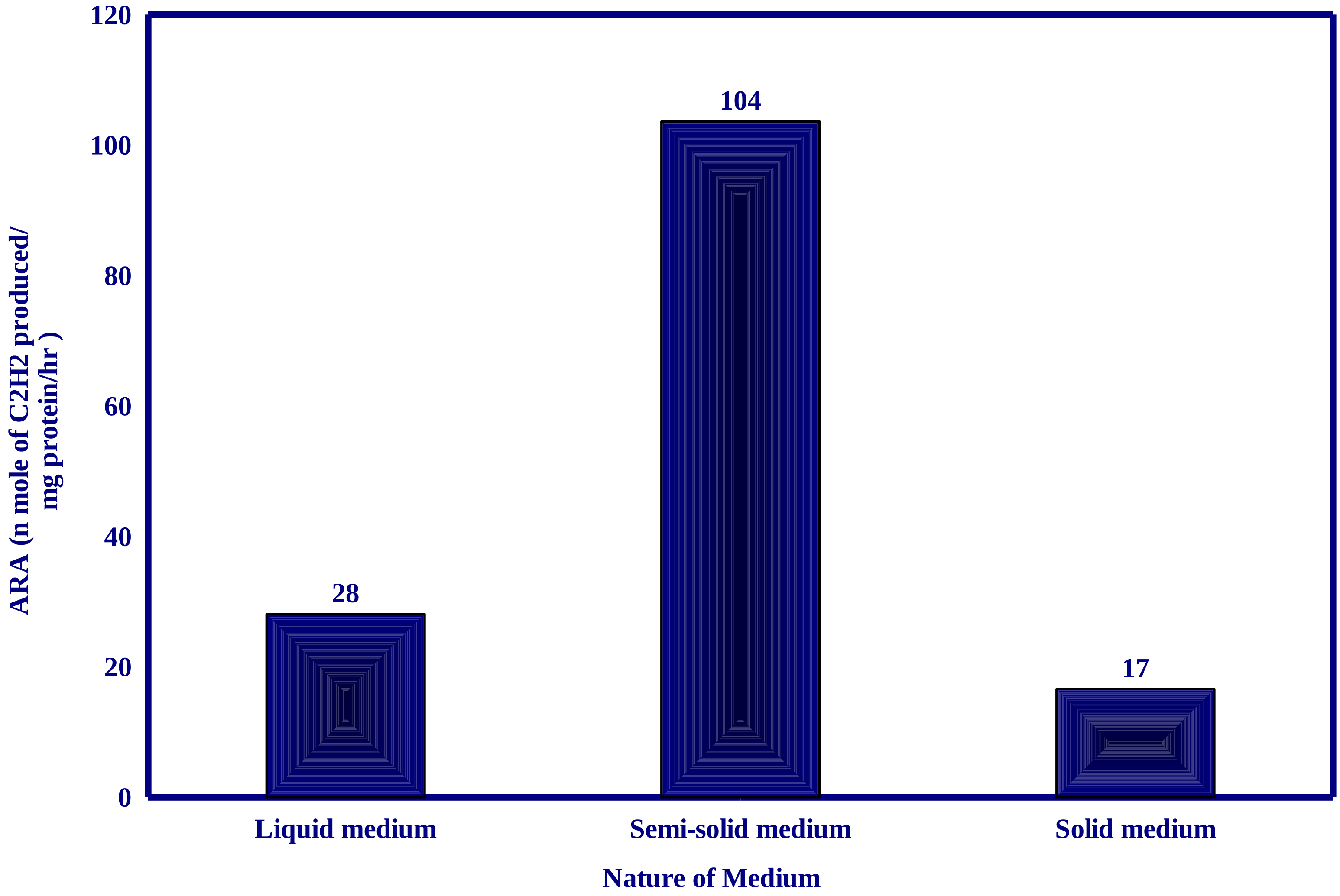


Fig 11:Impact of Azospirillum population on nitrogenase activity

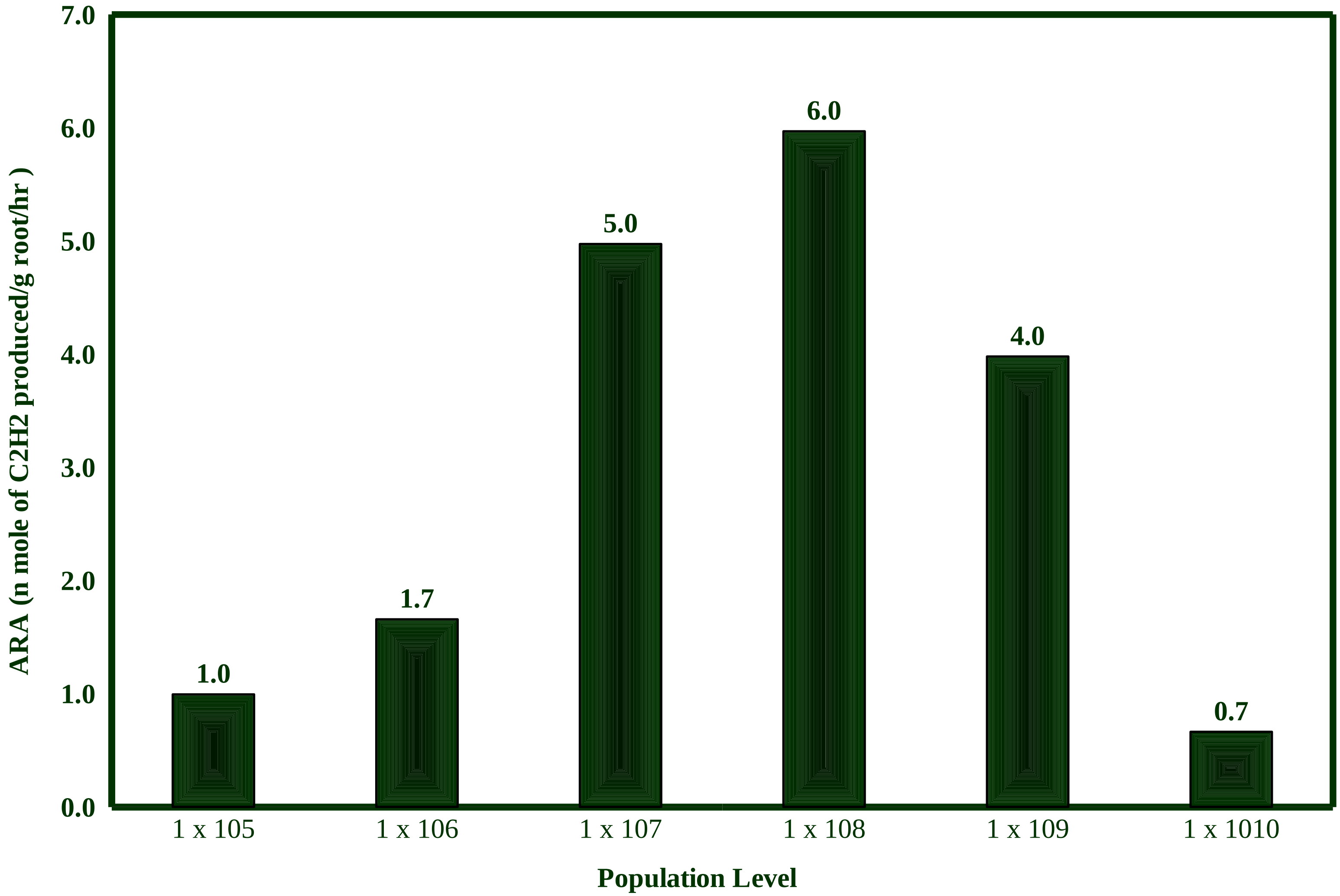
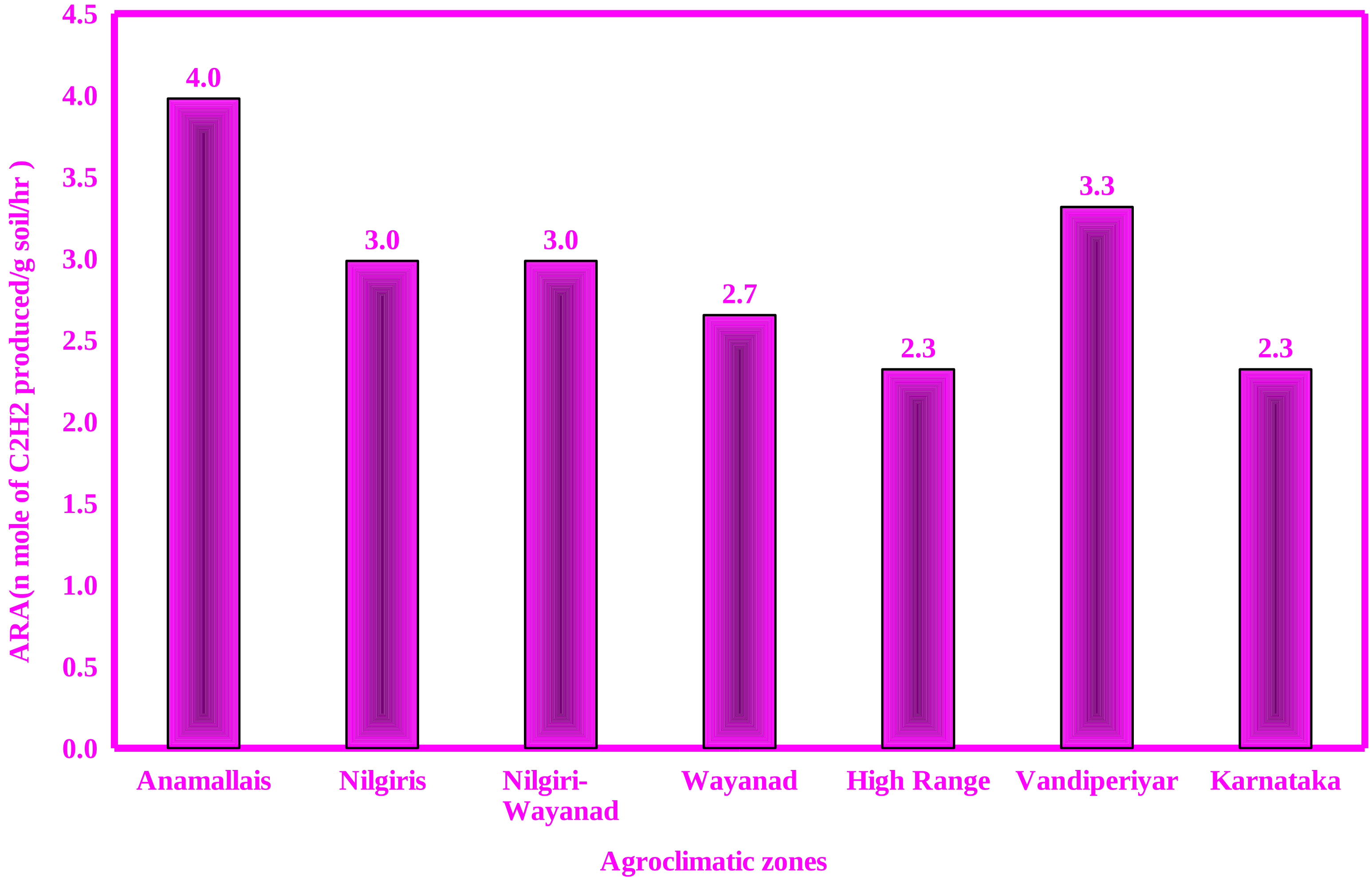


Fig 12: Nitrogenase activity in soil samples inoculated with Azospirillum strains of the respective regions



g 13: Nitrogenase activity in Anamallais soil inoculated with Azospirillum strains of different regio

Fig 14: Effect of Azospirilluminoculation on nitrogenase activity of root samples

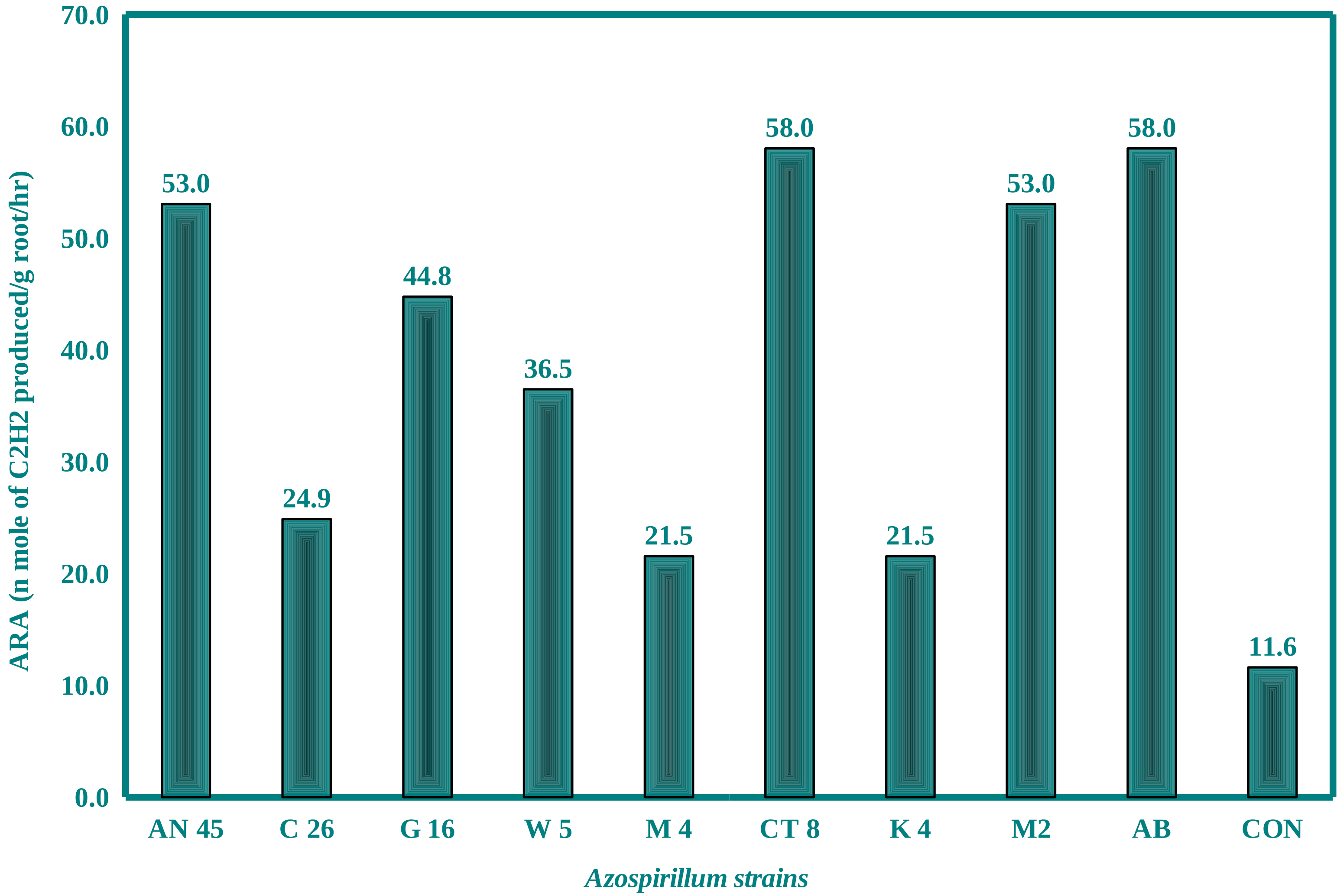


Fig 15: Effect of agrochemicals on nitrogenase activity

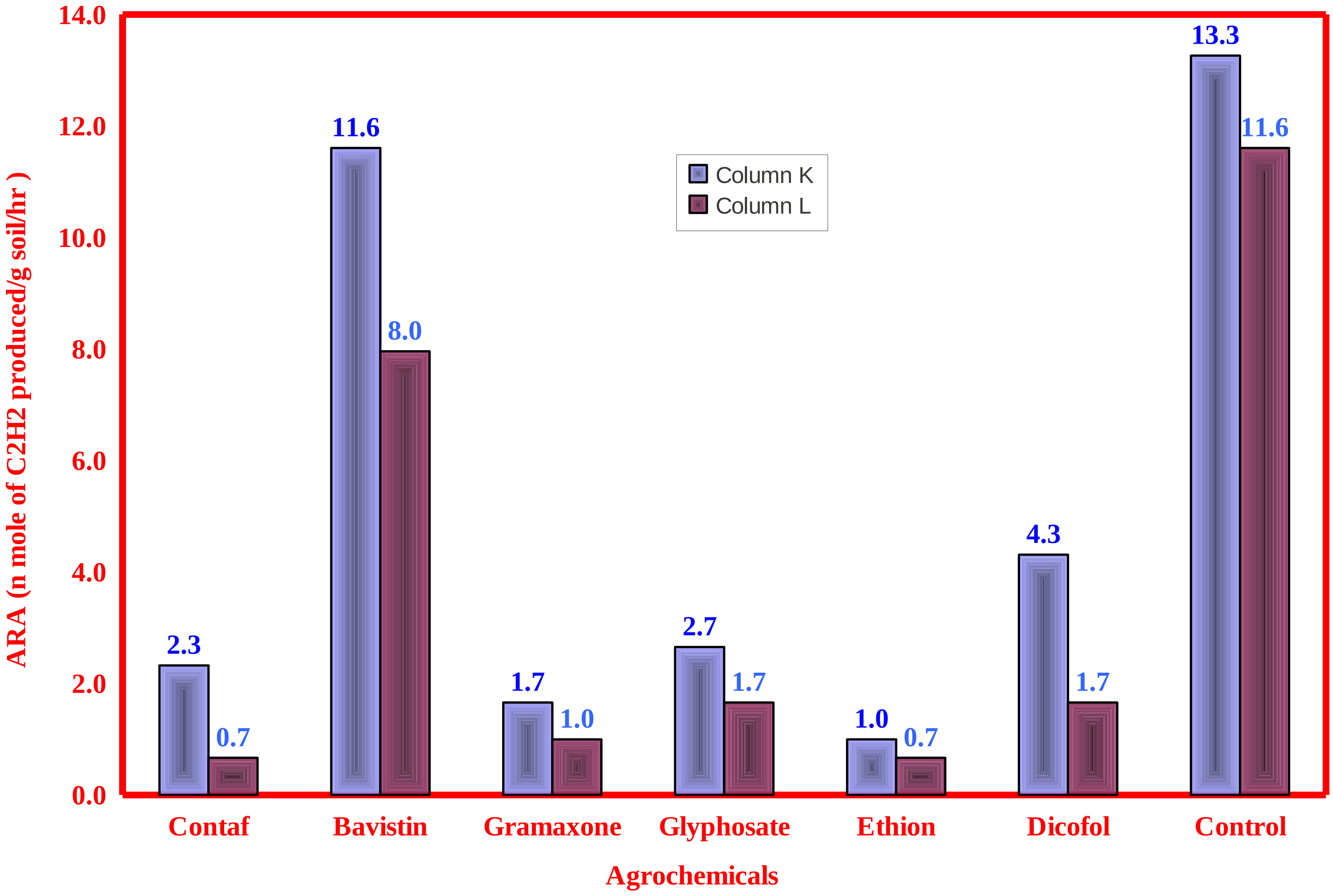


Fig 16: Growth kinetics

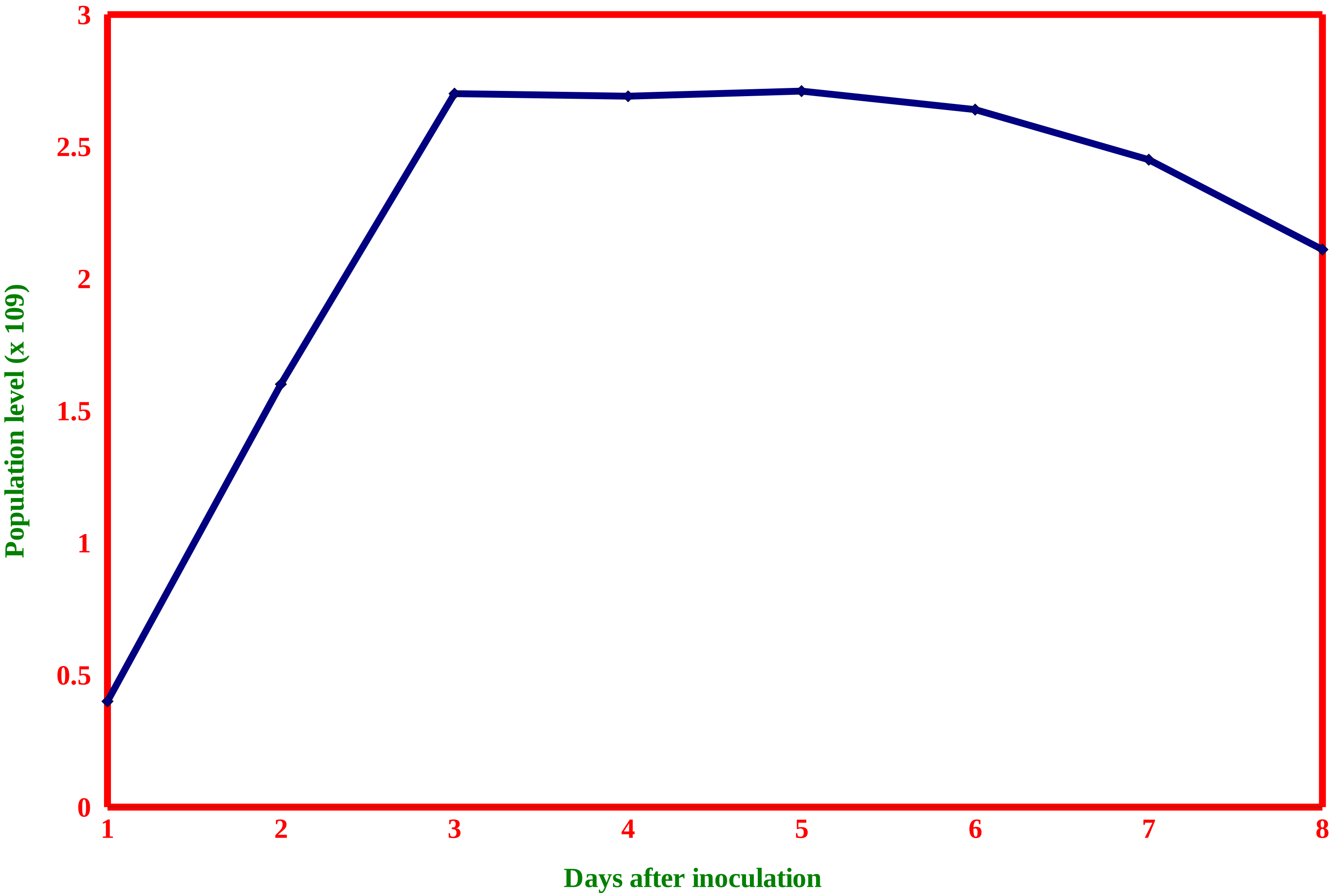


Fig 17: Population level of Azospirillum in carrier materials

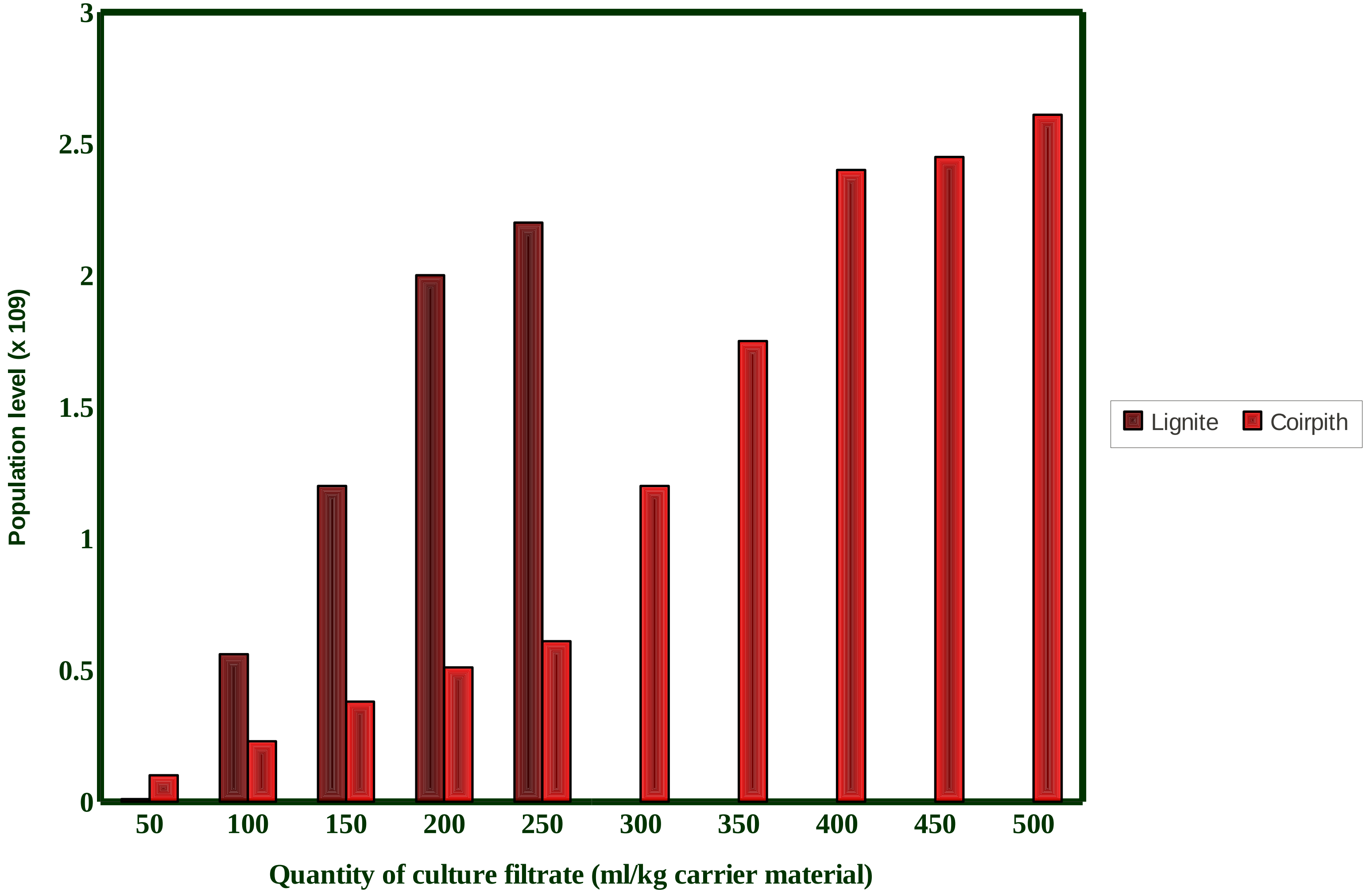
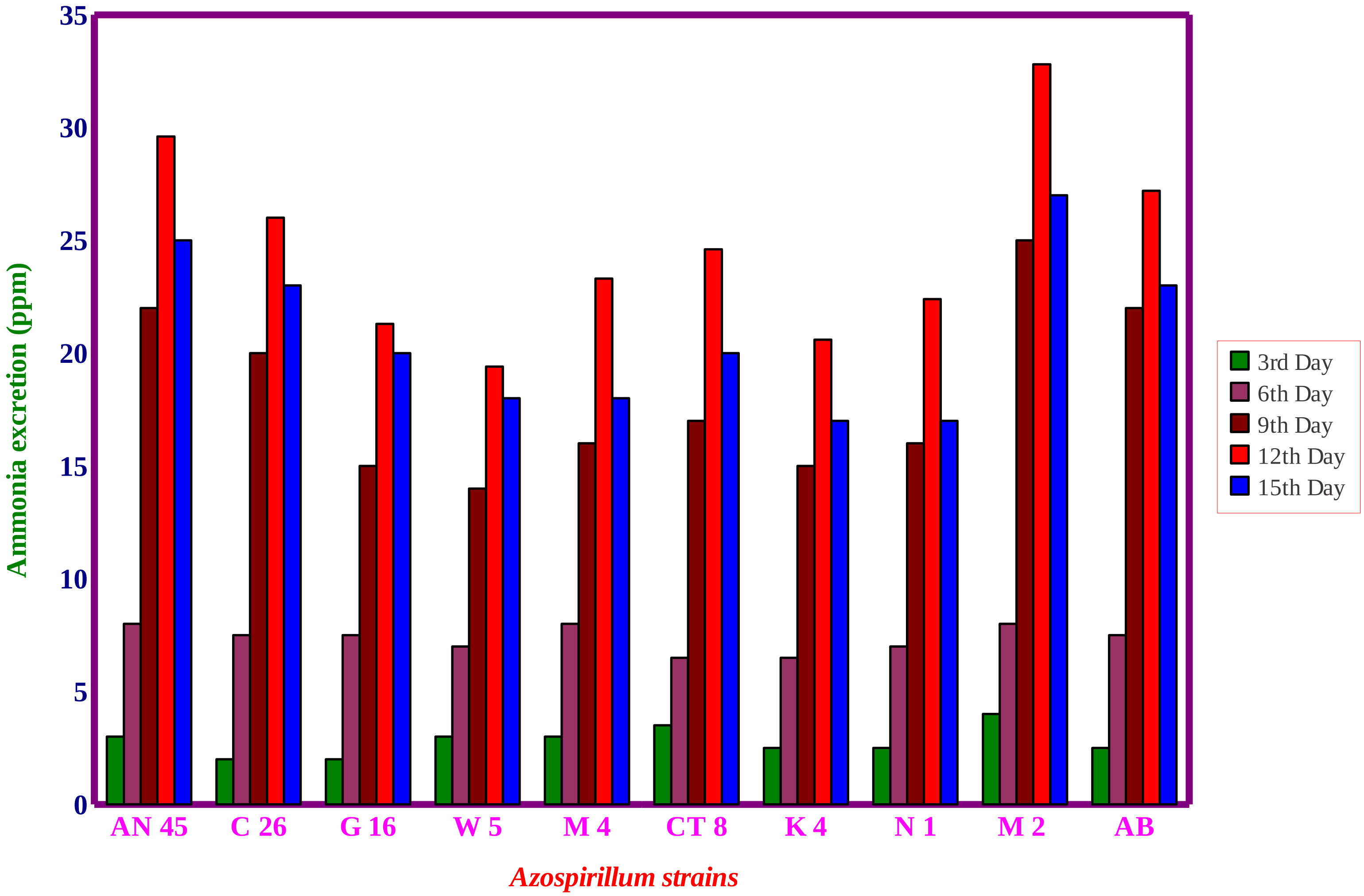


Fig 4: Ammonia excretion by Azospirillum strains



Nitrogenase activity

	Sample	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Annamallais	32.0	5.0	32	2.59	2.7	53.0
2	Nilgiris	15.0	5.0	32	2.59	1.2	24.9
3	Nilgiris-Wayanad	27.0	5.0	32	2.59	2.2	44.8
4	Wayanad	22.0	5.0	32	2.59	1.8	36.5
5	HighRange	13.0	5.0	32	2.59	1.1	21.5
6	Vandiperiyar	35.0	5.0	32	2.59	2.9	58.0
7	Karnataka	13.0	5.0	32	2.59	1.1	21.5
8	M 2	32.0	5.0	32	2.59	2.7	53.0
9	AB	35.0	5.0	32	2.59	2.9	58.0
10	Control	7.0	5.0	32	2.59	0.6	11.6

	Sample	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Annamallais	10.0	25.0	32	2.59	0.8	3.3
2	Coonoor	5.0	25.0	32	2.59	0.4	1.7
3	Gudalur	5.0	25.0	32	2.59	0.4	1.7
4	Wynaad	7.0	25.0	32	2.59	0.6	2.3
5	Munnar	5.0	25.0	32	2.59	0.4	1.7
6	C.travancore	10.0	25.0	32	2.59	0.8	3.3
7	Karnataka	5.0	25.0	32	2.59	0.4	1.7
8	M 2	9.0	25.0	32	2.59	0.7	3.0
9	AB	9.0	25.0	32	2.59	0.7	3.0
10	Control	1.0	25.0	32	2.59	0.1	0.3

Population

		Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Soil+1g	3.0	25.0	32	2.59	0.2	1.0
2	Soil+2g	5.0	25.0	32	2.59	0.4	1.7
3	Soil+3g	15.0	25.0	32	2.59	1.2	5.0
4	Soil+4g	18.0	25.0	32	2.59	1.5	6.0
5	Soil+5g	12.0	25.0	32	2.59	1.0	4.0
6	Control	2.0	25.0	32	2.59	0.2	0.7

RC soil

		Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Anamallais	12.0	25.0	32	2.59	1.0	4.0
2	Coonoor	9.0	25.0	32	2.59	0.7	3.0
3	Gudalur	9.0	25.0	32	2.59	0.7	3.0
4	Wynaad	8.0	25.0	32	2.59	0.7	2.7
5	Munnar	7.0	25.0	32	2.59	0.6	2.3
6	C.travancore	10.0	25.0	32	2.59	0.8	3.3
7	Karnataka	7.0	25.0	32	2.59	0.6	2.3

Fungicide I

	Chemical	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Contaf	7.0	25.0	32	2.59	0.6	2.3
2	Bavistin	35.0	25.0	32	2.59	2.9	11.6
3	Gramaxone	5.0	25.0	32	2.59	0.4	1.7
4	Glyphosate	8.0	25.0	32	2.59	0.7	2.7
5	Ethion	3.0	25.0	32	2.59	0.2	1.0
6	Dicofol	13.0	25.0	32	2.59	1.1	4.3
7	Control	40.0	25.0	32	2.59	3.3	13.3

Fungicide II

	Chemical	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)	
1	Contaf	2.0	25.0	32	2.59	0.2	0.7	
2	Bavistin	24.0	25.0	32	2.59	2.0	8.0	
3	Gramaxone	3.0	25.0	32	2.59	0.2	1.0	
4	Glyphosate	5.0	25.0	32	2.59	0.4	1.7	
5	Ethion	2.0	25.0	32	2.59	0.2	0.7	
6	Dicofol	5.0	25.0	32	2.59	0.4	1.7	
7	Control	35.0	25.0	32	2.59	2.9	11.6	

Root-all strains		
	NRA(n moles of C2H2 produced / mg of protein/hr)	
AN 45	53.0	
C 26	24.9	
G 16	44.8	
W 5	36.5	
M 4	21.5	
CT 8	58.0	
K 4	21.5	
M2	53.0	
AB	58.0	
CON	11.6	

Soil-all strains		
AN 45	3.3	
C 26	1.7	
G 16	1.7	
W 5	2.3	
M 4	1.7	
CT 8	3.3	
K 4	1.7	
M2	3.0	
AB	3.0	
CON	0.3	

1 x 10^5	1.0	
1 x 10^6	1.7	
1 x 10^7	5.0	
1 x 10^8	6.0	
1 x 10^9	4.0	
1 x 10^{10}	0.7	
RC soil		
Anamallais	4.0	
Nilgiris	3.0	
Nilgiri-Wayanad	3.0	
Wayanad	2.7	
High Range	2.3	
Vandiperiyar	3.3	
Karnataka	2.3	
Contaf	2.3	0.7
Bavistin	11.6	8.0
Gramaxone	1.7	1.0
Glyphosate	2.7	1.7
Ethion	1.0	0.7
Dicofol	4.3	1.7
Control	13.3	11.6

	Lignite	Coirpith		1	0.4	0.01
50	0.01	0.1		2	1.6	0.56
100	0.56	0.23		3	2.7	2.11
150	1.2	0.38		4	2.69	2.09
200	2	0.51		5	2.71	2
250	2.2	0.61		6	2.64	1.91
300		1.2	1.11	7	2.45	1.75
350		1.75	1.22	8	2.11	
400		2.4	1.25	9	1.64	
450		2.45	1.54	10	1.1	
500		2.61	1.67			

0.67
11.84
17.21

1	20	100
2	20	100
3	20	100
4	20	100
5	20	100
6	20	100
7	20	100

Azospiril	**3rd Day**	**6th Day**	**9th Day**	**12th Day**	**15th Day**
AN 45	3	8	22	29.6	25
C 26	2	7.5	20	26	23
G 16	2	7.5	15	21.3	20
W 5	3	7	14	19.4	18
M 4	3	8	16	23.3	18
CT 8	3.5	6.5	17	24.6	20
K 4	2.5	6.5	15	20.6	17
N 1	2.5	7	16	22.4	17
M 2	4	8	25	32.8	27
AB	2.5	7.5	22	27.2	23

Nitrogenase activity

pH

	pH	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)	
1	3.5	16.0	2.9	32	2.59	1.3	45.7	
2	4.0	18.0	3.0	32	2.59	1.5	50.5	
3	4.5	98.0	8.4	32	2.59	8.1	96.8	
4	5.0	88.0	8.0	32	2.59	7.3	91.4	
5	5.5	68.0	6.1	32	2.59	5.6	92.5	
6	6.0	60.0	5.0	32	2.59	5.0	99.5	
7	6.5	62.0	5.1	32	2.59	5.1	100.8	
8	7.0	40.0	6.3	32	2.59	3.3	52.7	
9	7.5	13.0	5.9	32	2.59	1.1	18.3	
10	8.0	10.0	4.9	32	2.59	0.8	16.9	
11	8.5	6.0	3.8	32	2.59	0.5	12.9	
12	9.0	4.0	3.0	32	2.59	0.3	11.2	

Temp

	pH	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)	
1	5.0	14.0	5.9	32	2.59	1.2	19.7	5.0
2	10.0	32.0	5.9	32	2.59	2.7	45.0	10.0
3	20.0	75.0	6.5	32	2.59	6.2	95.6	20.0
4	30.0	75.0	5.9	32	2.59	6.2	105.4	30.0
5	40.0	77.0	7.4	32	2.59	6.4	86.2	40.0
6	50.0	2.0	1.9	32	2.59	0.2	8.7	50.0

Days

1	1	21.0	4.0	32	2.59	1.7	43.5
2	2	60.0	6.5	32	2.59	5.0	76.5
3	3	75.0	5.8	32	2.59	6.2	108.0
4	4	50.0	6.0	32	2.59	4.1	68.7
5	5	30.0	4.9	32	2.59	2.5	50.8

Carbon

	Carbon sources	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Glucose	20.0	4.9	32	2.59	1.7	33.8
2	Fructose	50.0	4.9	32	2.59	4.1	84.5
3	Lactose	30.0	5.1	32	2.59	2.5	48.4
4	Maltose	85.0	6.6	32	2.59	7.0	107.0
5	Sucrose	60.0	5.3	32	2.59	5.0	93.9
6	Strach	30.0	4.9	32	2.59	2.5	50.8
7	Cellulose	35.0	5.1	32	2.59	2.9	56.9
8	Malate	90.0	5.9	32	2.59	7.5	126.6
9	Succinate	75.0	6.1	32	2.59	6.2	102.0
10	Lactate	41.0	5.3	32	2.59	3.4	64.2
11	Citrate	55.0	4.9	32	2.59	4.6	92.9
12	Mannitol	20.0	4.5	32	2.59	1.7	36.7
13	Galactose	17.0	3.9	32	2.59	1.4	35.7

Nitrogen

	Nitrogen sources	Peak height (mm)	Cell protein (mg/ml)	attenu-ation	Std graph	**n moles of ethylene**	NRA(n moles of C2H2 produced / mg of protein/hr)
1	Amm.chloride	50.0	6.3	32	2.59	4.1	65.8
2	Pot.nitrate	30.0	4.9	32	2.59	2.5	50.7
3	Amm.nitrate	33.0	5.0	32	2.59	2.7	54.7
4	Amm.sulphate	30.0	5.2	32	2.59	2.5	47.8
5	Urea	20.0	4.9	32	2.59	1.7	33.8
6	Control	85.0	5.9	32	2.59	7.0	119.4

Media

	Media						
1	Liquid medium	20.0	5.9	32	2.59	1.7	28.1
2	Semi-solid medium	75.0	6.0	32	2.59	6.2	103.6
3	Solid medium	10.0	5.0	32	2.59	0.8	16.6

3.5	45.7
4.0	50.5
4.5	96.8
5.0	91.4
5.5	92.5
6.0	99.5
6.5	100.8
7.0	52.7
7.5	18.3
8.0	16.9
8.5	12.9
9.0	11.2

19.7			5.9	5.9	10
45.0			7.4	6.5	20
95.6			8.1	5.9	30
105.4			10.1	7.0	40
86.2			4.9	5.9	5
8.7			1.9	1.9	50

1	43.5		(n moles of C2H2 produced /mg of protein/hr)
2	76.5		
3	108.0		
4	68.7		
5	50.8		

Glucose	33.8
Fructose	84.5
Lactose	48.4
Maltose	107.0
Sucrose	93.9
Strach	50.8
Cellulose	56.9
Malate	126.6
Succinate	102.0
Lactate	64.2
Citrate	92.9
Mannitol	36.7
Galactose	35.7

6.3

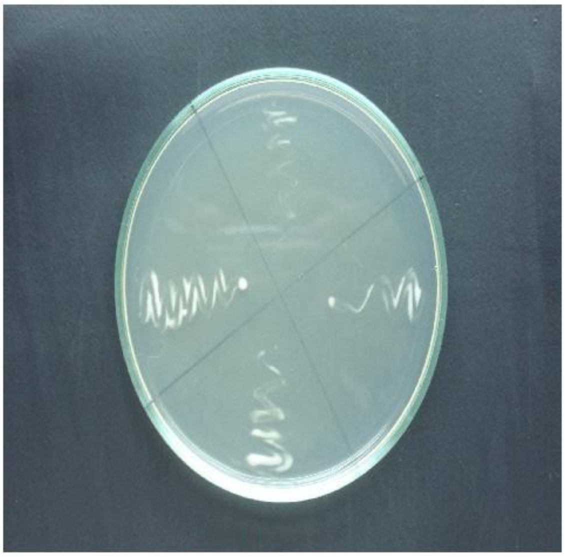
Amm.chloride	65.8
Pot.nitrate	50.7
Amm.nitrate	54.7
Amm.sulphate	47.8
Urea	33.8
Control	119.4

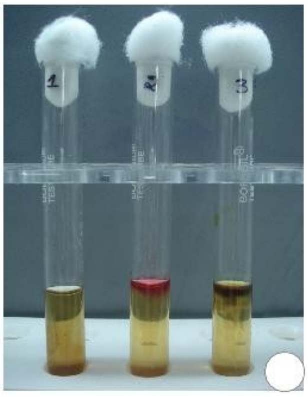
				mg protein/ml of LC
Liquid medium	28.1			5.9
Semi-solid medium	103.6			6.0
Solid medium	16.6			5.0

AN 45	121.2
C 26	43.3
G 16	110.4
W5	114.4
M 4	42.3

CT 8	157.3							
K 4	68.6							
M 2	65.6							
AB	62.7							

	4.9
	5.0
	5.2
	4.9
	5.9

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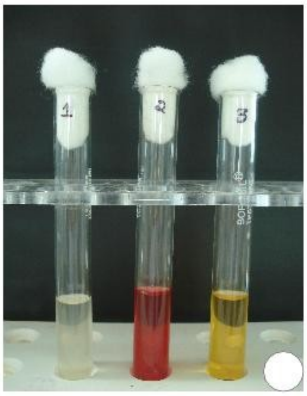
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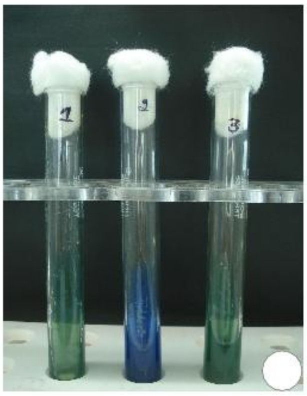
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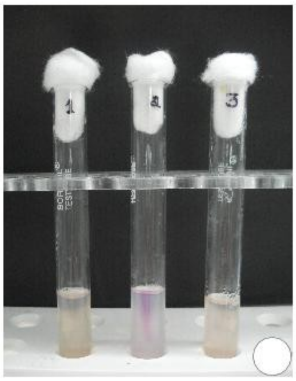
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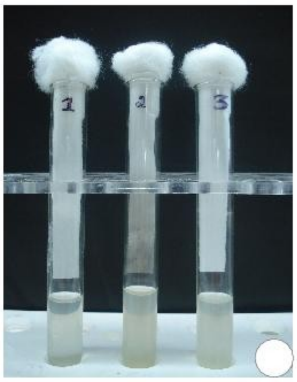
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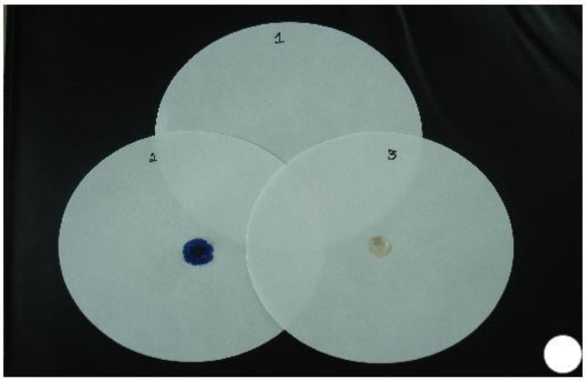
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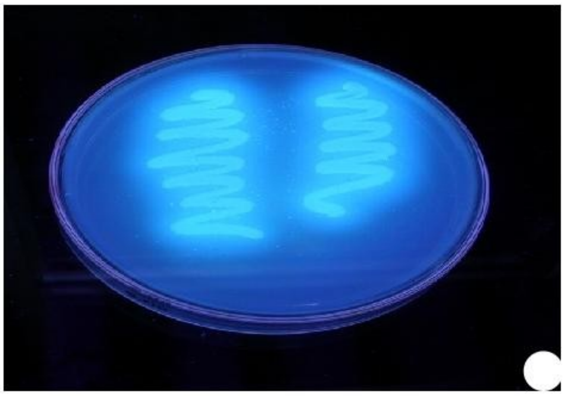

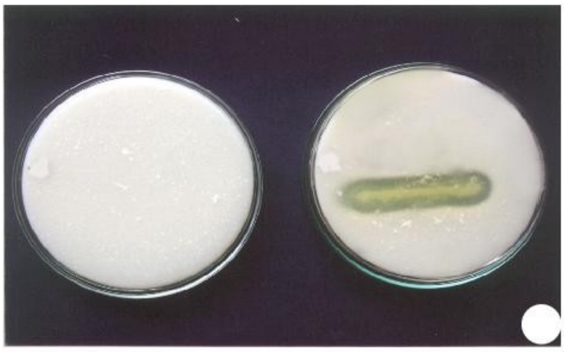

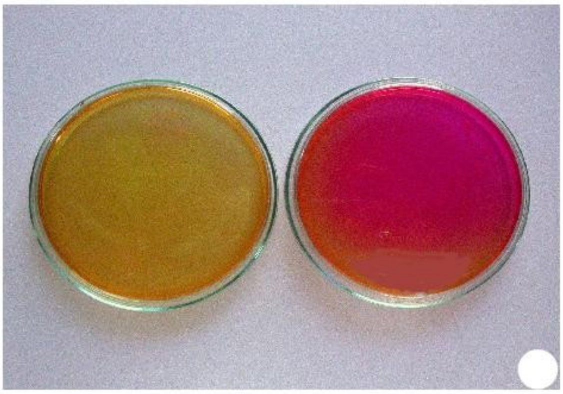

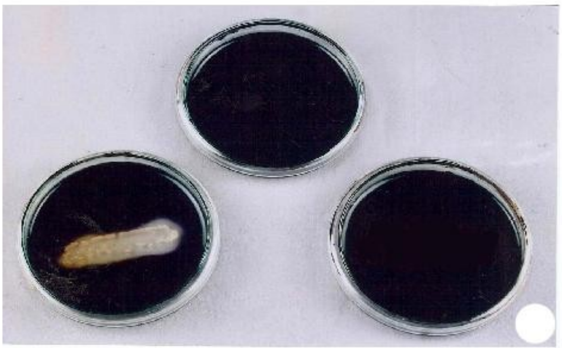


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