ASSESSMENT OF FUNCTIONAL MICROBIAL DIVERSITY IN THE RHIZOSPHERE OF SELECTED SEMI-ARID TROPICAL GRASSES

Thesis submitted in part fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY

to the Tamil Nadu Agricultural University, Coimbatore

By

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ID. No. 07-805-002

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

AGRICULTURAL COLLEGE AND RESEARCH INSTITUTE

TAMIL NADU AGRICULTURAL UNIVERSITY

COIMBATORE – 641 003

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CERTIFICATE

This is to certify that the thesis entitled "**ASSESSMENT OF FUNCTIONAL MICROBIAL DIVERSITY IN THE RHIZOSPHERE OF SELECTED SEMI-ARID TROPICAL GRASSES**" submitted in part fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE) IN AGRICULTURAL MICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Mrs. SARATHAMBAL, C.** under my supervision and guidance and that no part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles. However, part of the work has been published in peer reviewed scientific journal of national/international repute (copy enclosed).

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(Sarathambal, C).

Abstract

ABSTRACT

ASSESSMENT OF FUNCTIONAL MICROBIAL DIVERSITY IN THE RHIZOSPHERE OF SELECTED SEMI-ARID TROPICAL GRASSES

By

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Biological nitrogen fixation by plant-associated bacteria is eco-friendly and has been effectively exploited for crop plants including legumes. Although associations of diazotrophic bacteria with non-leguminous plants such as grasses have been known for decades, they have been poorly - studied. Weedy grass species normally thrive in adverse conditions and act as potential habitats for the diverse groups of elite bacteria with multiple beneficial characters remains unexplored. A more complete understanding of the diversity and functioning of diazotrophic microorganisms, especially those that have symbiotic relationships with grass species is of great value for agricultural research and application. Hence, the present investigation is focused to identify and characterize the unexplored culturable rhizospheric and endophytic diazotrophic bacterial diversity of selected grass species of India.

Based on their dominance in each physiological region of the country, a total of 10 different grass species (*Brachiaria reptans, Cenchrus glaucus, Saccharum spontaneum, Panicum repens, Cyperus rotundus, Dactyloctenium aegyptium, Chloris barbata, Oryza rufipogon, Cyanodon dactylon* and *Setaria verticillata*) along with rhizosphere soils were selected for the present investigation. The physio-chemical and biological properties of samples analysed and showed significant difference among the regions.

A total of 60 diazotrophic isolates from rhizosphere and 21 putative endophytic isolates were obtained from 10 grasses. A total of 30 rhizospheric and 20 endophytic diazotrophic isolates were selected based on their growth on N- free medium, total nitrogen content in the medium and ammonia excretion. Among the isolates, highest nitrogenase activity were exhibited by isolate OR3 (172.25 ±13.95 n moles of ethylene mg^{-1} of protein h^{-1}) isolated from *Oryza rufipogon.* Among the endophytic diazotrophs, highest ARA activity was found in CGE3 (171.45 ±11.80 n moles of ethylene mg^{-1} of protein h^{-1}) isolated from *Cenchrus glaucus*. The intrinsic antibiotic resistance and carbon-substrate utilization profiling of the selected isolates showed remarkable difference among them, forming seven different clusters.

The 16S rRNA gene sequence homology revealed the presence of diversity of γ Proteobacteria and Firmicutes. The authenticated diazotrophic isolates of grass species were screened for the presence of *nifH* gene and the amplification could be clearly detected in 15 isolates. Plant growth promoting traits of all the diazotrophs isolates from grass species were analysed. Among the rhizosphere diazotrophic isolates, the amount of IAA and GA production, P-solubilization, siderophore and HCN production were higher in *Serratia marcescens* (CD1). Likewise, among the endophytic diazotrophic isolates, *Pseudomonas* sp. (CRE10) showed maximum multiple- plant growth promoting traits. Seven rhizospheric and five endophytic isolates produced ACC deaminase and thereby regulated the ethylene concentration. Most of the selected diazotrophic isolates showed antagonistic activity against sheath blight (*Rhizoctonia solani)*, blast (*Pyricularia oryzae*) and sheath rot (*Sarocladium oryzae*) of rice.

Tolerance level to various abiotic stresses such as salt, temperature, dessication and heavy metals were assessed and the results revealed that these rhizosphere isolates *viz*., *Bacillus* sp. (CG5), *Stenotrophomonas* sp. (SS4) and *Bacillus* sp. (CD2) and the endophytic isolates *viz*., *Enterobacter sacchari* (SVE9), *Bacillus* sp. (CBE9) and *Serratia* sp. (CRE9) registered their remarkable ability to tolerate salt upto 7.5%. Thirty two percent of the isolates were found to survive at 55ºC. The moisture potentials (field capacity and permanent wilting point) were not found to significantly influence the population of diazotrophs. The diazotrophic isolates *Bacillus* sp. (CG5), *Bacillus* sp.

(CBE9) and *E. sacchari* (SVE9) showed the maximum resistance to heavy metals such as Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Under gnotobiotic conditions, *Pseudomonas* sp. (CRE10) inoculation registered maximum shoot length and root length in rice. Plant dry matter production was higher in *S. marcescens* (CD1) than the other isolates. The endophytic colonization in the rice seedlings (roots and culm) were examined by SEM analysis. Results indicated that endophytic bacteria were preferentially colonized in the rhizoplane and inner side of rice roots.

A field experiment was carried out to evaluate the efficiency of elite isolates on the growth and yield of rice (cultivar- ADT 43) under lowland ecosystem. Among the diazotrophic isolates tested, *Serratia* sp. (CB2) isolated from *Chloris barbata* enhanced plant growth, productive tiller counts, nitrogen uptake, arginine deaminase activity and fluorescein diacetate hydrolysis activity. *Klebsiella pneumoniae* (CR2) isolated from *Cyperus rotundus* and *Serratia* sp. (CB2) registered grain yield increase of 32 and 31% respectively compared to standard bioinoculant treatment.

The present investigation, unravel the diversity richness of diazotrophic bacteria colonizing in the rhizospheric and internal tissues of naturally growing weedy grass species in different parts of India. The present work suggest exploring of these elite diazotrophic strains having multiple plant growth promoting traits, as bioinoculants for nutrient management and for biotic and abiotic stress mitigation and sustainable crop production with fewer chemical inputs.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Abbreviations

ABBREVIATIONS

A	-	Ampicillin
ADA	-	Arginine deaminase activity
At	-	Azithromycin
Au	-	Augmentin
BAz	-	Azelaic acid medium
BR	-	*Brachiaria reptans*
CB	-	*Chloris barbata*
CD	-	*Cyanodon dactylon*
Ce	-	Cephotaxime
Cf	-	Ciprofloxacin
CG	-	*Cenchrus glaucus*
Cj	-	Cefaclor
Cq	-	Cephadroxyl
CR	-	*Cyperus rotundus*
Cs	-	Cefaperazone
Cu	-	Cefuroxime
Cw	-	Clariththromycin
DA	-	*Dactyloctenium aegyptium*
DM	-	Dry matter production
E	-	Erythromycin
ET	-	Effective tillers
FDA	-	Fluorescein diacetate
GY	-	Grain yield
H	-	Height
JNFb	-	Junior N free bromothymol blue medium
N	-	Nitrogen uptake
Nfb	-	N free bromothymol blue medium
NFMM	-	N free malic acid medium

OR	-	*Oryza rufipogon*
P	-	Penicillin
pH	-	Hydrogen ion concentration
PL	-	Panicle length
ppm	-	Parts per million
PR	-	*Panicum repens*
RL	-	Root length
rpm	-	Revolutions per minute
SE	-	Standard Error
SL	-	Shoot length
SM	-	Semi solid malate medium
SS	-	*Saccharum spontaneum*
SSM	-	Soil saturation extract semi solid malate medium
SV	-	*Setaria verticillata*
SY	-	Straw yield
T	-	Number of tillers
TBE	-	Tris-borate-EDTA Buffer
TEMED	-	N, N, N', N' - Tetramethyl ethylene diamine
TW		Test weight
UV	-	Ultraviolet
V/V	-	Volume by volume
w/v	-	Weight by volume
^{o}E	-	East
^{o}N	-	North
%	-	Per cent
°C	-	Degree Celsius
µg	-	Microgram
µl	-	Microlitre
µmol	-	Micro molar

Introduction

CHAPTER I

INTRODUCTION

In the context of increasing international concern for food security and environmental quality, the use of bioinoculants like diazotrophs and plant growth-promoting rhizobacteria (PGPR) for reducing chemical inputs in agriculture is a potentially important issue. The improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, where both rely on soil biological processes and soil biodiversity.

Nitrogen fixed biologically by plant-symbiotic bacteria is ecologically friendly and has been effectively exploited for important leguminous crop species. Although associations of diazotrophic bacteria with non-leguminous plants such as grasses have been known for decades (Dobereiner, 1992), they have been less studied in other crop plants except for a few cases; for example, associative bacteria of some tropical species of rice and maize (Reis *et al.*, 2000). A more complete understanding of the diversity and function of diazotrophic microorganisms, especially those that have clear relationships with commercially important non-leguminous plant species is of great value for research and application. The rhizosphere microbiology of native plants is important in view of the *in situ* conservation of the biodiversity associated with such niches to sustain delicate ecological processes in the oligotrophic ecosystem. Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N poor environments (Cocking, 2005).

Most plants in their native environments depend on interactions with microorganisms for their existence. Endophytic bacteria ubiquitously inhabit most plant species and have been isolated from a variety of plants. Recently, it has been reported that endophytic bacteria may promote plant growth and suppress the plant diseases probably by means similar to plant growth-promoting rhizobacteria (PGPR). Therefore, a better understanding of endophytic bacteria may help to elucidate their functions and potential role more effectively in developing sustainable systems of crop production. The search for natural association and endophytic interaction of diazotrophs with grass species is considered very promising, especially in grasses that grow naturally

with adverse environmental conditions. Evidence of significant biological nitrogen (N_2) fixation in economically important graminaceous species, particularly sugar cane (*Saccharum* sp.), rice (*Oryza sativa*) and forage grasses, such as kallar grass (*Leptochloa fusca*) has generated tremendous interest in endophytic N_2 fixation by non-legumes and over the last few years research in the area of biological nitrogen fixation (BNF) associated with cereals and grasses. These plants are considered excellent hosts to identify superior endophytes that may potentially impact the crop growth (Reinhold-Hurek and Hurek, 1998).

The search for diverse plant growth-promoting (PGP) diazotrophic bacteria is gaining momentum as efforts are made to exploit them as bioinoculants for various economically important crops. The knowledge on the diversity of diazotrophic bacteria is required not only for understanding their ecological importance but also for their utilization in sustainable agricultural as inoculants of rice. There is now increasing evidence that the use of beneficial microbes can enhance plant's resistance to adverse environmental stresses, e.g., drought, salts, nutrient deficiency and heavy metal contaminations (Glick *et al.*, 2007).

With this information, the present investigation hypothesizes that the grass species grown in different physiographic regions do harbour potential microbes and shows lot of scope for the identification of novel functional microbes. Accordingly it is planned to screen native population from the selected grass species rhizosphere for the isolation of nitrogen fixing, plant growth promoting and mineral solubilizing bacteria. The grass species are obtained from various physiographic regions of India.

To address the above hypothesis, the present investigation was taken up with the following objectives.

- Isolation of culturable diazotrophs from rhizosphere and internal tissues of grass species obtained from various physiographic regions of India.
- Phylogenic characterization and diversity analysis of isolated diazotrophs by 16S rRNA gene sequencing.
- Assessment of plant growth promoting activity and tolerance to various abiotic stress *viz.*, temperature, desiccation, heavy metals etc.
- Crop response study to evaluate the efficiency of elite bacterial cultures.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Microbial communities are a fundamental component of ecosystems that play critical roles in metabolism of organic matter and in biogeochemical transformations of elements, including nitrogen fixation. Beneficial Plant-microbial interactions in the rhizosphere interactions can be divided into three categories (Brimecombe *et al.*, 2001). The first interaction includes microorganisms which, in association with the plant, increase the supply of mineral nutrients to the plant. This is the case of the symbiotic dinitrogen-fixing bacteria of leguminous plants and the free nitrogen fixing bacteria such as *Klebsiella pneumoniae* (Lugtenberg *et al.*, 1991). Secondly, there are microorganisms like fluorescent pseudomonads that stimulate plant growth indirectly by the production of antibiotics, siderophores, volatile compounds or hydrolytic enzymes which prevent the growth or activity of plant pathogens. Nowadays, these bacteria are used as biocontrol agents. Thirdly, there are the plant growth-promoting rhizobacteria that stimulate directly plant growth by the production of phytohormones (Okon, 1985). Detrimental interactions within the rhizosphere involve deleterious rhizobacteria which inhibit shoot or root growth without causing any other visual symptoms by the production of phytotoxins such as cyanide (Alstrorn and Burns, 1989) or phytohormones (Schippers *et al.*, 1987). Biological nitrogen fixation is an important source of fixed nitrogen for the biosphere. Most of plants in their native environments depend on symbioses with microorganisms for their existence. Nitrogen fixed biologically by plant-symbiotic bacteria has been effectively exploited for important leguminous crop species (Dobereiner, 1977). Although associations of diazotrophic bacteria with non-leguminous plants such as grasses have been known for decades, they have been less studied in other crop plants except for a few cases; for example, associative bacteria of some tropical species of rice and maize (Cocking, 2005).

Soil microorganisms play an important role in soil processes that determine plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitats. The diversity of microorganism in agro-ecosystem is critical to the management of good

soil health because they are involved in many important soil processes. Diversity and community structure in the rhizosphere is however influenced by both, plant and soil type. The association of grasses with diazotrophic bacteria in Indian soil is poorly understood. Plant-species-specific selective enrichment of microflora in the rhizosphere milieu has been exploited in legumes from the point of view of N_2-fixation under nitrogen limiting conditions (Coutinho, *et al.*, 1999). Likewise, non-leguminous crops also favour specific bacterial groups in its rhizosphere. Nitrogen fixation is one of the essential beneficial biological processes for the economic and environmental sustainability of agriculture worldwide. Globally, annual inputs of fixed nitrogen from crop legume–rhizobia symbioses are estimated as 2.95 million tonnes for pulses and 18.5 million tonnes for oilseed legumes (Howieson, 2005). In spite of the in-depth knowledge about the biochemical and molecular steps involved in legume-rhizobium symbiosis, the holy grail of N_2 fixation by non-legumes, especially cereal food crops, is yet to be realised. It is essential to enhance the activities of microbes that benefit plant nutrition, control diseases and assist plants to cope with a variety of abiotic stresses to sustain and improve global food production in future climate scenarios while maintaining environmental health. A diverse range of beneficial microorganisms have been found but their reliable use in field environments is yet to be fully realised. New knowledge on soil microbial diversity can lead to the discovery of new generation inoculants as well as improve survival and performance of beneficial microbes in situ following their introduction into foreign environments.

A more complete understanding of the diversity and function of diazotrophic microorganisms, especially those that have symbiotic relationships with non-leguminous plant species particularly plant experiencing abiotic stress, is of great value for agricultural application. Some recent progress in this field of diazotrophs associated with different grasses and cereals was reviewed in this chapter.

2.1. Diversity of microbiota in soil

Soil biota plays a vital role in the maintenance of soil fertility and productivity and soil microorganisms drive most soil processes like nutrient availability and retention, decomposition of organic material, soil organic matter buildup and stabilization of soil

aggregates. The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. Microbial diversity in soil influenced by different factors including anthropogenic activities and microbial communities are known to respond to organic matter amendments with increased activity and growth which affects soil processes including nitrogen mineralization. Environmental conditions such as temperature, pH, availability of nutrients and habitat complexity affect the microbial diversity of particular region.

As the biodiversity of an ecosystem increases, the resilience and stability of the ecosystem should increase. Conversely, as ecosystems degrade, ecosystem biodiversity decreases (Garbeva *et al.*, 2004). Loss of biodiversity leads to loss of ecosystem resistance and resilience to anthropogenic as well as natural stresses (Van Elsas *et al.*, 2002). Microorganisms exhibit an impressive diversity in their metabolic activities and in their interactions with other microbes, plants and animals (Nannipieri *et al.*, 2003). Microbes have much shorter turnover rates than higher plants and therefore, respond more quickly to changes in land management than plants and may be the most sensitive indicator to anthropogenic activities. In the past it has been nearly impossible to discern underlying patterns within ecosystems because of the intrinsic structural and functional diversity of microbial communities. To date, only 1–5 per cent of the world's microorganisms have been identified (Hugenholtz *et al.*, 1998).

One major function of a soil is its use as a habitat for soil organisms including maintaining of biodiversity, assemblage, and activity for both soil microflora and fauna. Soil microorganisms themselves are involved in major soil processes, such as humification, recycling and mineralization of organic residues, leading to the plant availability of nutrients. The mechanical fragmentation of organic residues, stabilization of soil aggregates, or bioturbation and mixing of organic and mineral substances are governed primarily by soil animals. In turn, these activities positively influence the physico-chemical properties of soil and consequently soil fertility and quality. In the case of cultivated soils, every single farming practice may influence soil fertility and quality status either in a positive or negative manner. In terrestrial ecosystems, soil organisms play an essential role in the cycling of elements (mineralization and humification) and stabilization of soil structure. The mineralization of organic matter is carried out by a

large community of organisms and involves a wide range of metabolic processes. Most of these processes are mediated by soil enzymes which are produced by soil microorganisms, roots and to some extent by soil animals. Therefore, the composition of soil biota determines the potential of the community for enzyme synthesis. The actual rate of enzyme production and the fate of produced and sorbed enzymes are modified by environmental effects and ecological interactions (Klose *et al.*, 1999).

2.2 Rhizosphere microbial diversity

In the field soil, nutrient cycling, plant root and biological activity are found in the top 20-40 cm, called rooting zone. The rhizosphere is characterized as a zone of interest microbial activity and represents the close interaction among the plants, soil and soil microorganisms. The rhizosphere is enriched in energy rich carbon compounds, leaked photosynthates from plant roots including sugars, amino acids and organic acids. All plant species exudates unique composition of compounds from their roots that determines the microbial community of that rhizosphere. In the battle for the unique compounds released, bacterial strains produces antibiotics and other compounds that remove the competition (some time help plant to have lesser deleterious organisms in their rhizosphere) and /or production of plant growth promoting substances that increases root growth, ultimately increases the volume of root area available for and increasing the root exudates (Fig .1). In total the rhizosphere is a partnership between plant, soil and soil microorganisms.

Plant roots can stimulate or inhibit microbial populations and their activities through the exudation of different compounds. Root exudates are water-soluble organic compounds, mainly carbohydrates, organic acids and amino acids, released from the root cells along concentration gradients in the rhizosphere soil (Lynch and Whipps, 1990). For microorganisms, these exudates represent a convenient source of carbon (and possibly nitrogen) since they are readily assimilated without the need to synthesize exo-enzymes. Due to this large availability of substrates in the rhizosphere, microbial biomass and activity are generally much higher in the rhizosphere than in the bulk soil (Brimecombe *et al.*, 2001). The release of carbon in form of root exudates may account for up to 40% of the dry matter produced by plants (Lynch and Whipps, 1990). Even if the

C-transfer to exudation was 10-20% of total net fixed carbon other microbial symbionts such as mycorrhizae (Lemanceau *et al.*, 1995) or N_2-fixing microorganisms may each consume another 10-20 % of total net fixed carbon, so that plants would still release up to half of their total fixed carbon to fuel microbial interactions in the rhizosphere. Supporting microbial interactions in the rhizosphere must be of fundamental importance for plants to justify this significant input of carbon, which could otherwise be used (Fig. 1). Moreover, the quantity and the chemical composition of root exudates, which vary during plant developmental stage and between plant species (Brimecombe *et al.*, 2001) may also affect the microbial community structure in the rhizosphere. This was confirmed by the results of several studies using cultivation (Germida *et al.*, 1998) or molecular fingerprinting techniques (Grayston *et al.*, 1998 and Kuske *et al.*, 2002). According to the key role that root exudates seem to play in determining the composition of their associated rhizobacterial populations, they could potentially be used in the future for the remediation of contaminated sites by the selective enhancement of certain bacterial populations which might improve the heavy metal uptake by the plant (Kozdroj and van Elsas, 2000).

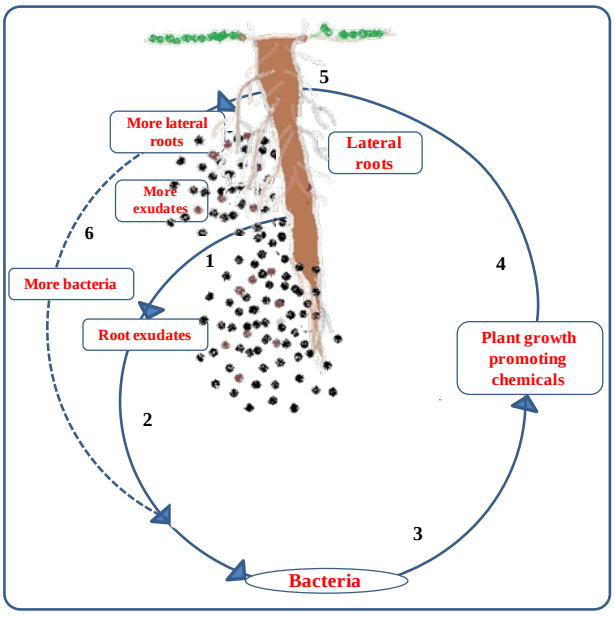

Fig.1. A conceptual model illustrating root exudates-induced hormonal effects on root growth. Root exudation (1) stimulates growth of a diverse bacterial community (2) and subsequently favours plant hormone producing bacteria (3) and hormonal release induces lateral growth (4), the release of more exudates (6) subsequent bacterial growth, etc.

2.3. Diversity of diazotrophs associated with grasses and cereals

"Diazotrophy", the ability to fix atmospheric nitrogen catalysed by the enzyme nitrogenase, is distributed among diverse groups of bacteria and archaea (Reed, 2011). Free-living N_2 fixing bacteria (for example, *Azospirillum* spp., *Azotobacter* spp., *Acetobacter diazotrophicus, Herbaspirillum* spp., *Bacillus* spp., *Azoarcus* sp.) are found in the rhizosphere and rhizoplane environments of cereal crops. Recent evidence not only identified new genera of N_2 fixing bacteria and archaea in natural and managed ecosystems but also indicated significant edaphic and environmental groupings in genetic diversity and functionality (Wakelin, 2011). Non-rhizobial N_2 fixing bacteria can grow as endophytes in a number of grasses, for example, in a recent study in South Australia *Pseudomonas* species were the most dominant group of *nifH* carrying bacteria found in the rhizosphere of perennial native grasses (Gupta *et al.*, 2011). Evidence suggests the *nifH* gene is present in a number of non-Frankia actinobacteria (for example, *Agromyces*, *Microbacterium*, *Corynebacterium* and *Micromonospora*). Thus the challenge is to identify (i) functionally significant N_2 fixing genera/species specific to biomes and crops, and (ii) key edaphic and environmental drivers regulating the genetic diversity and free living N_2 fixation in order to maximise benefits from these beneficial microbes both for sustainable primary production and climate change adaptation.

Evidence of significant biological nitrogen (N_2) fixation in economically important gramineous species, particularly sugar cane (*Saccharum* sp.), rice (*Oryza sativa*) and forage grasses, such as kallar grass (*Leptochloa fusca*) has generated tremendous interest in N_2 fixation by non-legumes and over the last few years research in the area of biological nitrogen fixation (BNF) associated with cereals and grasses has become divided into two areas. On the one hand there have been a large number of reports of responses of field-grown plants to inoculation with N_2-fixing bacteria, principally *Azospirillum* spp. This organism came into focus with the work of Dobereiner and associates from Brazil. Bagwel *et al*.(1998) reported geochemical variable can adversely affect plant productivity and spatial distribution resulting strong zonation of plant species and growth forms. This geochemically induced stress may also influence the species

compilation and distribution of rhizosphere diazotrophs assemblages, but little is currently known about these organisms. 339 Gram negative strains were isolated from salt marsh grasses and 72% of them are diazotrophs.

On the other hand there have been several reports of significant contributions of associated BNF to the nutrition of several crops, including wetland rice, sugar cane and some forage grasses. The recent discovery of many more plant-associated N_2-fixing bacteria suggests that further research in this area may eventually lead to the development of such associations with applications for agricultural productivity. In Brazil, a number of tropical forage grasses, including *Brachiaria humidicola*, *B. decumbens*, *Paspalum notatum* and *Panicum maximum* have shown relatively high N_2 fixation rates in ^{15}N isotope dilution studies, and may derive up to 40% of their N-needs from fixation (Olivares *et al.*, 1996). High nitrogen fixation by kallar grass in Pakistan has also been reported by Malik *et al.* (1997).

Gluconoacetobacter diazotropicus isolated from roots and stems of sugarcane was first reported as an N_2-fixing bacterium from Brazil, and subsequently from Australia, India, Mexico, Uruguay, Canada and Cuba. Isolation of this bacterium from most tissues of sugarcane, and its absence from the soils of sugarcane fields suggested these to be systemic endophytes. The occurrence of this organism has been reported in sugar-rich plants like *Pennisetum purpureum* and sweet potato (Paula *et al.*, 1991) and in insects like mealybugs and leafhoppers (Vadivelu *et al.*, 1996). The colonization of *A. diazotropicus* has also been reported in coffee plants grown through seeds and vegetative propagation. This bacterium successfully colonizes sugarcane varieties in India where the chemical N fertilization is completely avoided for at least two successive years and replaced by organic manures. *Acetobacter* has gained importance as an inoculant for sugarcane (Salgado *et al.*, 1997). The family Acetobacteriaceae includes genera, *Acetobacter*, *Gluconobacter*, *Gluconoacetobacter* and *Acidomonas* (Yamada *et al.*, 1998). Based on 16S rRNA sequence analysis, the name *Acetobacter diazotrophicus* has been changed to *Gluconoacetobacter diazotrophicus*. In addition to *G. diazotrophicus*, two more diazotrophs, *G. johannae* and *G. azotocaptans* have been included in the list. The genetic diversity of *G. diazotrophicus* isolated from various sources does not exhibit much variation. However, Suman *et al.* (2001) found that the diversity of the

isolates of *G. diazotrophicus* by RAPD analysis was more conspicuous than that reported on the basis of morphological and biochemical characters. The SDS-PAGE and multilocus enzyme electrophoresis analysis also revealed certain differences among strains of *G. diazotrophicus* suggesting genotypic differences. On the basis of DNA fingerprinting studies, existence of genetically distinct *G. diazotrophicus* strains in sugarcane cultivars has been reported from Louisiana (Liang and Damann., 1998). Investigations of isolates of *G. diazotrophicus* from pineapple suggested that only certain genetically related groups of this bacterium or its ancestors have acquired the capability of colonizing plants by themselves or with the aid of the vectors such as insects or fungi (Tapia-Hernadez *et al.*, 2002). Verma *et al.* (2004) reported that endophytic occurrence of the members of Enterobacteriaceae is more consistent than others; interestingly, they were conspicuous by their absence in the soils/sediments of the lake in which deep water rice varieties grew. This strongly indicated that members of Enterobacteriaceae are transmitted from one generation of rice to the next, not by the contact of seeds with soil, but directly via seeds in a manner similar to seed-borne pathogens. Most of these endophytic bacteria produced IAA, pectinase and cellulase that would help to invade plant tissues. Some were able to solubilize insoluble phosphate but only *Pantoea*, *Citrobacter* and *Klebsiella* possessed the ability to fix atmospheric nitrogen (Verma *et al.*, 2001). As Enterobacteriaceae are known to fix nitrogen anaerobically, it was logical that the submerged portions of rice under the deepwater facing nearly anaerobic condition may be the right locations for endophytic nitrogen fixation. One of the diazotrophic endophytes, i.e. *Pantoea* was genetically tagged with both gus- and gfp-reporters, and shown to vigorously colonize the inter-cellular spaces in the roots of the rice seedlings (Verma *et al.*, 2004).

The discovery of novel nodulating groups, classified within the β-proteobacteria are based on the analysis of the 16S rRNA gene sequences. These sequences constitute the largest database for the comparison of new isolates. The *Burkholderia* genus currently contains five named rhizobial members and others as *Burkholderia* sp. which has been reported to nodulate many legumes. Twenty Mimosa-nodulating bacterial strains from Brazil and Venezuela, are examined by amplified rRNA gene restriction analysis (Chen *et al.*, 2005). All the isolates with 16 patterns formed a single cluster together with the known beta-rhizobia, *B. caribensis*, *B. phymatum*, and *B. tuberum*. The 16S rRNA gene

sequences of 15 of the 20 strains are determined, and all are shown to belong to the genus *Burkholderia*; four distinct clusters could be distinguished, with strains isolated from the same host species usually clustering very closely.

Enterobacteria, including diazotrophs have been isolated from a considerable range of plants, especially members of graminae but also from various dicots including trees. For rice, diazotrophic enterobacteria have been isolated from many different varieties of grown under wetland, dryland and deep water conditions. They can be isolated from all parts of the plant, including the rhizosphcrc, spermosphere, leaf sheath, stem, phyllosphere and seeds as well as the inner and surface portions of surface and non-surface-sterilized roots. So far all enterobacters isolated from rice belong to the genera *Enterobacter, Klebsiella* and *Serratia*. Interestingly, diazotrophic enterobacteria were also detected in *Monochoria vaginalis,* a wetland plant frequently associated with paddy fields (Barraquio and Watanabe, 1981). These data show that diazotrophic enterobacters are ubiquitous in rice soil and that they possess the ability to compete in a setting full or other microorganisms. They also suggest the likelihood of a close relationship between diazotrophic enterobacteria and rice. The density of the enterobacterial population in the rhizosphere of rice typically ranges from 10^6 to $l0^8$ bacteria g^{-1} dry weight of soil (Balandreau, 2002). Though a speculative idea, it seems plausible that long use in agriculture of farmyard manures for crop may have developed a flora well adopted to an animal - soil - plant rhizosphere nutritional cycles.

Effect of salinity on the diversity of two important plant associated bacteria, i.e. *Azospirillum* and *Pseudomonas,* is investigated at several paddy fields with varying levels of salinity. An increase in salinity led to decrease in bacterial diversity. PCR-RFLP of 16S rDNA from 256 *Pseudomonas* strains isolated from five paddy cultivation sites revealed the occurrence of 18 different genotypes. Fluorescent *Pseudomonads* dominated at non-saline sites whereas salt-tolerant species, in particular *Pseudomonas alcaligenes* and *P. pseudoalcaligenes* dominated the saline sites. Diversity of *Pseudomonads* at saline sites was higher when organic farming was practiced, showing positive effects of organic farming on the diversity of *Pseudomonads* under saline conditions (Rangarajan *et al.*, 2002). Taxonomic analysis of 402 strains isolated by enrichment in NFB medium from 12 paddy cultivation sites with varying salinity and soil texture revealed that 302 of them belonged

to *Azospirillum*. They were represented by 19 fingerprints (genotypes) based on PCR-RFLP of 16S rDNA. Of the 19 genotypes, 15 were specific to non-saline soils whereas only two genotypes were specific to saline soils. Identification based on nucleotide sequence of 16S rDNA revealed that the bacterial community in the rice rhizosphere from salt-affected rice consisted of *Alacaligenes xylosoxidans*, *Ochrobactrum anthropi*, *Serratia marcescens* and *Pseudomonas aeruginosa*. Loganathanan and Nair, (2004) reported the novel genus, *Swaminathania salitolerans* from salt-tolerant, mangrove-associated wild rice (*Porteresia coarctata*). This novel bacterium was able to fix nitrogen and solubilize phosphate in the presence of NaCl.

2.4. Endophytic bacteria and their potential applications

'Endophyte' is derived from the Greek 'endon'(within) and 'phyte' (plant), and until recently this term had usually been applied to fungi (Carroll, 1988 and Clay, 1988), including the mycorrhizal fungi (O'Dell and Trappe, 1992). However, for the purpose of this review the definition of endophyte will include 'fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease' (Wilson, 1995).

When physical barriers are overcome by invading bacteria, a selective enrichment of certain genotypes of bacteria inside the plant and thus a lower diversity than in the surrounding soil will be achieved. Therefore, microorganisms living endophytically will face less competition for nutrients. Another advantage of living inside a plant might be a partial protection from changes in the environment, such as osmotic stress due to lowered water activities. A well-studied example is the legume-nodule symbiosis, in which the bacteria fix nitrogen as endosymbionts inside the plant, in a nutrient-rich, oxygen controlled micro-environment (Mylona *et al.,* 1995). Therefore, the emphasis is on endophytic bacteria in several groups searching for diazotrophs that might be able to contribute to the nitrogen requirements of gramineous plants (Dobereiner *et al.,* 1993). Gramineous plants differ in their capacity to support associated nitrogen fixation. For example, breeding of sugar cane, which is shown to support biological nitrogen fixation, traditionally grown without large amounts of N-fertilizer. Moreover, nitrogen fixation

may be variety-specific, depending on the plant genome only some of the Brazilian sugar cane cultivars tested gained high amounts of their nitrogen content from biological nitrogen fixation in uninoculated Brazilian soils. Studies using ^{15}N-dilution techniques also demonstrated the dependence of N_2-fixation on the variety of rice (Wu *et al.*, 2006). Plants that are likely to support biological nitrogen fixation are among the first that are screened successfully for the presence of diazotrophic endophytic bacteria, such as sugar cane (Cavalcante and Döbereiner, 1988) or Kallar grass (Reinhold *et al.*, 1986). Kallar grass (*Leptochloa fusca* (L.) Kunth) is a C4-plant that is widely distributed in tropical and subtropical regions ranging from Australia to Africa. Due to its tolerance to soil salinity and waterlogged conditions, this plant has been used for soil reclamation in the Punjab of Pakistan. On salt affected, saline-sodic, low-fertility soil, 20 to 40 metric tons of hay are harvested per ha per year without application of nitrogen fertilizer. A further indication for biological nitrogen fixation is the actelylene reduction activity associated with roots of this grass. Endophytes promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate, or contribute assimilable nitrogen to plants. Some endophytes are seedborne, but others have mechanisms to colonize the plants that are being studied (Table 1).

2.4.1. Mechanisms of Infection

Endophytes are capable of invading healthy plants with intact surfaces must overcome barriers to reach the plant interior. Cellulose is a major constitutent of cell walls of mature plant cells. The middle lamella between cells contains mainly pectin. Bacterial pectinases might therefore be important for an intercellular bacterial ingress into roots. It has been suggested that *Azospirillum* spp. invade the root by penetration of the middle lamella and at points of emergence of lateral roots. As pectinolytic activity was detected in *Azospirillum* spp., this might be an active process (Plazinski and Rolfe, 1985). However, pectinolytic activity has not yet been reported for diazotrophic endophytic bacteria. Pectinases may play an important role in plant-microbe interactions, as shown for plant-pathogenic bacteria. An opportunistic pathogen of market vegetables, *Pseudomonas viridiflava*, secretes a single pectate lyase that is required for pathogenicity. The widespread soft rot bacterium *Erwinia chrysanthemi* secretes complexes of several pectic enzymes. Individual mutations in these genes showed that they may vary in their

Table 1. Examples of reported bacterial endophytes and plants harboring them

Endophytes	Plant species	Reference
α Proteobacteria		
Azorhizobium caulinodans	Rice	Engelhard *et al*., 2000
Azospirillum brasilense	Banana, pineapple	Weber *et al*., 1999
Bradyrhizobium japonicum	Rice	Chantreuil *et al*. 2000
Gluconacetobacter diazotrophicus	Sugarcane, coffee	Cavalcante and Döbereiner ,1988
Rhizobium leguminosarum	Rice	Yanni *et al*.,1997
Rhizobium (*Agrobacterium*) *radiobacter*	Carrot, rice	Surette *et al*., 2003
β Proteobacteria		
Azoarcus sp.	Kallar grass, rice	Reinhold-Hurek *et al*.,1993
Burkholderia pickettii	Maize	McInroy and Kloepper, 1995
Burkholderia sp.	Banana, pineapple, rice	Weber *et al*. ,1999; Engelhard *et al*., 2000
Chromobacterium violaceum	Rice	Phillips *et al*. 2000
Herbaspirillum seropedicae	Sugarcane, rice, maize, sorghum, banana	Olivares *et al*., 1996; Weber *et al*. ,1999
Herbaspirillum rubrisulbalbicans	Sugarcane	Olivares *et al*., 1996
γ Proteobacteria		
Enterobacter spp.	Maize	McInroy and Kloepper ,1995
Citrobacter sp.	Banana	Martínez *et al*., 2003
Enterobacter sakazakii	Soybean	Kuklinsky-Sobral *et al*., 2004
Enterobacter cloacae	Citrus plants, maize	Araujo *et al*. 2002; Hinton *et al*., 1995
Klebsiella sp.	Wheat, sweet potato, rice	Iniguez *et al*. 2004; Reiter *et al*., 2003
Klebsiella pneumoniae	Soybean	Kuklinsky-Sobral *et al*., 2004
Klebsiella variicola	Banana, rice, maize, sugarcane	Rosenblueth *et al*., 2004
Stenotrophomonas	Dune grass	Dalton *et al*.,2004
Serratia marcescens	Rice	Gyaneshwar *et al*., 2001
Pseudomonas fluorescens	Carrot	Surette *et al*., 2003
Pantoea agglomerans	Citrus plants, sweet potato	Araujo *et al*.,2002; Asis and Adachi, 2003

Pantoea sp.	Rice	Verma *et al*., 2004
Firmicutes		
Bacillus spp.	Citrus plants	Araujo *et al*., 2001
Bacillus megaterium	Maize, carrot, citrus plants	Araujo *et al*.,2001;Surette *et al*.,2003
Paenibacillus odorifer	Sweet potato	Reiter *et al*.,2003
Sphingobacterium sp.	Rice	Phillips *et al*., 2000
Actinobacteria		
Arthrobacter globiformis	Maize	Chelius and Triplett , 2000
Curtobacterium flaccumfaciens	Citrus plants	Araujo *et al*., 2002
Kocuria varians	Marigold	Sturz and Kimpinski , 2004
Microbacterium testaceum	Maize	Zinniel *et al*., 2002

importance in different hosts. Moreover, the activity of pectic enzymes may release oligouronide products that elicit defense responses in plants. The microbial breakdown of another important plant polymer, cellulose, is a complex process to which at least three types of enzymes may contribute (Sharrock, 1988). An endo-1,4-β-glucanase (EC 3.2.1.4) cleaves internal β-1,4-glycosidic bonds, releasing large oligomers. Exo-1,4-cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) degrade oligo- or dimers, releasing mainly cellobiose or glucose, respectively. Cellulolytic enzymes have been studied up to date in *Azoarcus* spp. In most cellulolytic bacteria and fungi, cellulose degradation contributes directly to the supply of nutrients for growth. In contrast, the plant associated strains of *Azoarcus* neither grow on the breakdown products glucose or cellobiose nor on cellulose itself. Nevertheless, *Azoarcus* strain BH72 possesses a machinery for cellulose breakdown: an endoglucanase and an exoglycanase, which unspecifically releases small oligo- or monomers from oligomeric substrates (cellulose or xylane). Because the products are not catabolically utilized, this bacterium cannot truely be called cellulolytic. Also, several other features of this cellulolytic system suggest that it is used for a "mild infection" of the plant (Reinhold-Hurek *et al.,* 1993). Both enzymes are surface associated and not excreted into the culture medium; this might lead to a more localized reaction of and less damage to the plant than caused by plant pathogenic bacteria, which often secrete cellulases into the culture medium. The amino acid sequence deduced from the cloned endoglucanase gene of *Azoarcus* sp. had the highest homology to an endoglucanase of the phytopathogenic bacterium *Ralstonia* (formerly *Pseudomonas*) *solanacearum*. This suggests that the enzyme contributes to the process of monocot infection by *Azoarcus* sp. In the endosymbionts of legumes, rhizobia, endoglucanases are present and surface associated as in *Azoarcus* sp. There is indirect evidence that hydrolytic enzymes may be involved in the release of bacteria from the infection thread, or they might be involved in the invasion of root hairs. It has been observed that white clover, which is nodulated by *Rhizobium leguminosarum* via root hairs, shows a very localized disruption of the cristalline cellulose structure at the root hair tip, a change that is induced by a nodulation factor (Dazzo *et al.,* 1996). Mutational analysis has been carried out only for *Azorhizobium caulinodans,* which invades the host by crack entry: mutagenesis of the endoglucanase did not abolish the symbiotic phenotype. Therefore,

up to now *Azoarcus* sp. BH72 seems to be one example for the involvement of a cellulolytic enzyme in plant colonization by a diazotroph. Other plant-microbe interactions should be studied carefully to reveal if cellulases might be part of a common mechanism of invasion. A degradation of the plant cell wall to aid invasion might be their primary function. However, the breakdown products might as well elicit a plant response.

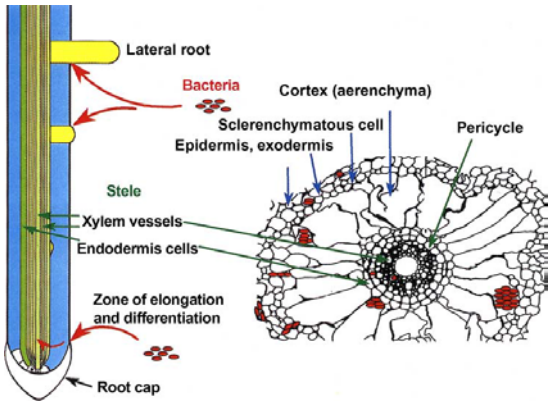

Fig. 2. Possible sites of colonization and infection of diazotrophic endophytes in roots, shown in a sketch of a longitudinal (left) and a transversal (right) section of rice roots. Adapted from Reinhold-Hurek and Hurek (1998).

Colonization sites are the rhizosphere soil (surrounding the roots, influenced by plant exudates) or, for epiphytic microorganisms, the rhizoplane (root surface), including dead cells of the outer cell layers (epidermis and exodermis), or inner tissues. In young roots or non-flooded plants, root cortex cells remain intact, whereas in flooded plants, such as rice and kallar grass, they lyse and are replaced by gas-filled spaces for aeration (aerenchyma). The endodermis is the barrier surrounding the central stele (green arrows), which contains xylem vessels for the transpiration stream and phloem vessels for nutrient supply of the root, and was previously thought not to be colonized by microorganisms other than pathogens.

2.4. Nitrogenase gene diversity

Nitrogen fixation is the key process of the global conversion of the atmospheric dinitrogen molecule to ammonia, which is catalyzed by the nitrogenase complex of prokaryotic microorganisms (Arp, 2000). For N_2- fixing bacteria, *nifH* (the gene coding

for dinitrogenase reductase, the small subunit of the nitrogenase complex) is well conserved in all diazotrophs and is therefore suitable for developing molecular tools to screen for the occurrence of nitrogenase in different habitats. The N_2 fixation requires the interaction of several gene products including the nitrogenase structural proteins like *nifD*, *nifK*, and *nifH*. The phylogeny based on *nifH* genes has been shown to resemble the 16S rRNA phylogeny (Zehr *et al.*, 2003); thus *nifH* is an ideal phylogenetic gene marker for investigating N_2-fixing organisms in natural environments. In the past, nifH gene has been successfully used to determine diversity of the diazotrophic communities (Wakelin *et al.*, 2007). Kirshtein *et al.* (1991) reported that by use of the polymerase chain reaction and degenerate oligonucleotide primers for highly conserved regions of *nifH*, segment of *nifH* DNA can be amplified from several microorganisms. This technique was shown to be useful for (i) the detection of N_2-fixing microorganisms and (ii) rapidly obtaining the DNA sequence of the *nifH* gene, which provides information about general taxonomic groups of N_2-fixing microorganisms.

Nitrogenase gene sequences (*nifH*) have been amplified and sequenced from a number of environments, including rice roots, soils, oceans and invertebrates, such as zooplankton and termites (Steppe *et al.*, 1996). However, the mere presence of nitrogenase genes does not indicate that bacteria are actively fixing nitrogen. Microorganisms catalyse biological nitrogen fixation with the enzyme nitrogenase, which has been highly conserved through evolution. Cloning and sequencing of one of the nitrogenase structural genes, *nifH*, has provided a large, rapidly expanding database of sequences from diverse terrestrial and aquatic environments. Comparison of *nifH* phylogenies to ribosomal RNA phylogenies from cultivated microorganisms shows little conclusive evidence of lateral gene transfer. Sequence diversity far outstrips representation by cultivated representatives. The phylogeny of nitrogenase includes branches that represent phylotypic groupings based on ribosomal RNA phylogeny, but also includes paralogous clades including the alternative, non-molybdenum, non-vanadium containing nitrogenases. Only a few alternative or archaeal nitrogenase sequences have as yet been obtained from the environment. Extensive analysis of the distribution of *nifH* phylotypes among habitats indicates that there are characteristic patterns of nitrogen fixing microorganisms in termite guts, sediment and soil environments, estuaries and salt marshes,

and oligotrophic oceans. Most microorganisms that perform biological N_2 fixation do so with an evolutionarily conserved nitrogenase protein complex (Howard and Rees, 1996).

nifH gene sequences from cultivated microorganisms, and their cluster affiliation are shown in Table 2 (Zehr *et al.*, 2003). The proteobacteria *nifH* sequences form a number of distinct clusters that correspond approximately, but not perfectly, with ribosomal RNA phylogeny, the gamma and alpha proteobacterial clusters are generally well-defined, although there are a few cultivated microorganisms that do not consistently cluster within the appropriate group. Cultivation techniques, although biasing for viable, cultivable cells, have provided crucial landmarks to ground truth sequences obtained by amplification (Steppe and Paerl, 2002). For example, the *nif* sequences of spirochaetes isolated from termites were very similar to sequences obtained by direct amplification (Lilburn *et al.*, 2001). Isolates have also been useful for developing *insitu* hybridization assays for enumerating and visualizing N_2- fixing cells *in situ* (Egener *et al.*, 1998). Archaeal nitrogenases have thus far only been found in methanogens, and though the presence of nitrogenase in other archaeal lineages is likely, it has yet to be documented. Archaeal nitrogenases include homologues of cyanobacteria are a morphologically and physiologically diverse group within the Bacteria. The cyanobacteria *nifH* genes cluster together (Zehr *et al.*, 1997) (Cluster 1B) although sequences from some unicellular and filamentous non-heterocystous cyanobacteria form deep branches. Heterocyst-forming cyanobacterial *nifH* form a tight cluster within the cyanobacterial group. Some cyanobacteria have a nitrogenase that is expressed in vegetative cells of filamentous heterocyst-forming species (Thiel *et al.*, 1995). Vanadium nitrogenases have also been reported in cyanobacteria and the *Anabaena variabilis* vnfH is found within the heterocyst-forming cyanobacterial *nifH* clade (Thiel, 1993). As most nitrogenases from cyanobacteria have not yet been characterized by mutant or deletion analysis, it is not yet possible to determine whether the vegetative, heterocyst or vnfH nitrogenases in cyanobacteria (Cluster 2) can be resolved by *nifH* phylogeny. Thus, the cyanobacterial *nifH* cluster contains *nifH* genes expressed in vegetative cells and heterocysts, as well as the cyanobacterial vnfH genes. The cyanobacteria gene cluster can intermingle with Frankia *nifH* sequences of the firmicutes (Hirsch *et al.*, 1995), although as more sequences have been added to the database, the resolution of these groups has improved.

Table 2. Phylogenetic distribution of *nifH* genes among cultivated microorganism (Zehr *et al* ., 2003).

Cluster	Group	Subcluster	Genera	Cluster	Group	Subcluster	Genera
I	Alpha	IJ	*Azospirillum*		Cyanobacteria	IB	*Azotobacter*
			Gluconacetobacter				*Anabaena*
			Mesorhizobium				*Chlorogloeopsis*
			Rhodobacter				*Calothrix*
			Rhodospirillum	I	Firmicutes	1D	*Frankia*
			Rhizobium			1E	*Paenibacillus*
			Sinorhizobium	II	Alpha	2C	*Rhodobacter*
		1K	*Beijerinckia*		Delta	2E	*Desulfobacter*
			Methylocella		Gamma	2C	*Azotobacter*
			Methylosinus		Firmicutes	2 D	*Paenibacillus*
			Methylocystis		Spirochaetes	2E	*Spirochaeta*
			Rhizobium		Archaea	2B	*Methanobrevibacter*
	Beta	IJ	*Burkholderia*				*Methanococcus*
		1K	*Burkholderia*				*Methanothermobacter*
		1P	*Herbaspirillum*	III	Delta	3B	*Desulfobacter*
		1U	*Azoarcus*			3E	*Desulfomicrobium*
			Alcaligenes			3J	*Desulfotomaculum*
	Epsilon	1F	*Arcobacter*		Firmicutes	3A	*Clostridium*
	Gamma	1H	*Vibrio*			3C	*Clostridium*
		1K	*Acidothiobacillus*		Spirochaetes	3C	*Spirochaeta*
		1l	*Klebsiella*		Archaea	3C	*Methanosarcina*
		1M	*Methylomonas*		Green sulphur	3L	*Chlorobium*
		1N	*Azotobacter (vnfH)*	IV	Spirochaetes	4A	*Treponema*
		1P	*Methylobacter*		Archaea	4	*Methanobrevibacter*
		1T	*Azomonas*				*Methanocaldococcus*
		1U	*Pseudomonas*			4D	*Methanosarcina*

Paenibacillus species are Gram-positive bacteria which are typically found in rhizosphere zones. Analysis of *nifH* genes in *Paenibacillus* species with a nested PCR approach showed that these organisms also contain multiple copies, with one homologue in Cluster II (Table 2) (Rosado *et al.*, 1998). The sequences from a number of cultivated diazotrophs are in Cluster III. These sequences include those from Gram-positive microorganisms, delta proteobacteria, green sulphur bacteria and Archaea. The organisms represented by *nifH* within this cluster are mostly, if not all, strict anaerobes. The known organisms in this cluster are *Clostridium*, *Desulfovibrio* (and other sulphate reducing genera), *Chlorobium*, and the archaea *Methanosarcina barkeri*. Although they cluster together, Cluster III is characterized by deep bifurcations and long branch lengths, and the distances between sequences are large relative to distances within Cluster I. The clustering of these sequences is not as aberrant with ribosomal phylogeny as it might first appear, as phylogenetic trees based on partial 16S ribosomal sequences from these microorganisms groups them together as well (Fig. 3). Sulphate reducers are known to be N_2 fixers and have nitrogenase genes (Kent *et al.*, 1989) that are in Cluster III. Experimental data implicated them in N_2 fixation in estuarine sediments (Dicker and Smith, 1980). However, it is curious that sulphate reducers fix N_2 in many cases, because they are active in highly reduced sediments where decomposition processes release ammonium. Steppe and Paerl (2002) investigated the involvement of sulphate reducers in nitrogen fixation in marine sediments and provided evidence for *nifH* gene expression. Cluster II and III *nifH* sequences from spirochaetes have been recently reported (Lilburn *et al.*, 2001) (Table 2). *nifH* genes from termites guts that are closely related to spirochaete *nifH* genes (Lilburn *et al.*, 2001) were shown to be transcribed using RT-PCR (Ohkuma *et al.*, 1999). Representative sequences for several major lineages are still not represented on the phylogenetic tree. For example, there are no nitrogenases from bacterial thermophiles (except from the green sulphur bacteria), yet it seems likely, given the dispersion of *nifH* through the prokaryotes, that they will be present in this group. Although it is possible that some of these lineages do not have N_2-fixing representatives, it is probably still true that we simply have not yet provided cultures with the right conditions to observe N_2-fixation. The high degree of similarity of protein sequence of nitrogenase among microorganisms suggests an early origin or lateral gene transfer among prokaryotic lineages.

As nitrogenase and 16S rRNA sequences accumulate in the databases, the high degree of sequence similarity supports early evolution of nitrogenase in an early ancestor (Young, 1992). Current research on a number of genes (Doolittle, 1999) and comparative genomics, however, once again raises the speculation that nitrogenases could have been dispersed by lateral gene transfer mechanisms. Further detailed comparative phylogenetic analyses will ultimately help resolve the relative evolutionary histories of nitrogenases and rRNA genes. Regardless of the end result, nitrogenase gene sequences provide us, at present, with a practical means of classifying and identifying uncultivated diazotrophic microorganisms. The distribution of nitrogen-fixing microorganisms, although not entirely dictated by the nitrogen availability in the environment, is non random and can be predicted on the basis of habitat characteristics. The ability to assay for gene expression and investigate genome arrangements provides (Zehr *et al.*, 2003). The diversity of diazotrophic bacteria associated with plants is directly detected based on *nifH* fragments without using a culture method. Albino *et al.* (2006) detected *nifH* DNA genes similar to those of *Bradyrhizobium* sp., *Klebsiella* sp., and *Serratia* sp. from mature sugarcane harvested on Tanegashima and Miyako Islands, Japan. Reiter *et al.* (2003) found a diversity of endophytic *nifH* genes in African sweet potatoes collected from farmers' fields in Uganda and Kenya. Hürek *et al.* (2002) reported that *Azoarcus* sp. inoculated on kallar grass (*Leptochloa fusca* L.) were not re-isolated, but *nifH* nucleotide sequence and *nifH* gene expression of *Azoarcus* sp. were detected by the PCR method. The biodiversity of diazotrophic bacteria within the soil, roots, and stems of field-grown maize was investigated using *nifH* gene sequences (Roesch *et al.* 2006).

2.5. Plant Growth-Promoting Effects of Diazotrophs

PGPR have the potential to contribute to sustainable plant growth promotion. Generally, PGPR function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the soil, and lessening or preventing the plants from diseases. Plant growth promotion and development can be facilitated both directly and indirectly. Indirect plant growth promotion includes the prevention of the deleterious effects of phytopathogenic organisms.

This can be achieved by the production of siderophores, *i.e.* small metal-binding molecules. Biological control of soil-borne plant pathogens and the synthesis of antibiotics have also been reported in several bacterial species. Another mechanism by which PGPR can inhibit phytopathogens is the production of hydrogen cyanide (HCN) and/or fungal cell wall degrading enzymes, e.g., chitinase and ß-1,3-glucanase. Direct plant growth promotion includes symbiotic and non-symbiotic PGPR which function through production of plant hormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid. Production of indole-3-ethanol or indole-3-acetic acid (IAA), the compounds belonging to auxins, have been reported for several bacterial genera. Some PGPR function as a sink for 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene in higher plants, by hydrolyzing it into α-ketobutyrate and ammonia, and in this way promote root growth by lowering indigenous ethylene levels in the micro-rhizo environment. PGPR also help in solubilization of mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates, and improve soil structure and organic matter content. PGPR retain more soil organic N, and other nutrients in the plant–soil system, thus reducing the need for fertilizer N and P and enhancing release of the nutrients.

2.5.1. Production of plant growth promoting substances

Phytohormones, also called plant growth regulators (PGRs), are well known for their regulatory role in plant growth and development. PGRs are organic substances that influence physiological processes of plants at extremely low concentrations. Because the concentration of hormonal signals is critical to the regulation of various physiological processes in plants, local changes of phytohormone levels can lead to characteristic changes in plant growth and development. In 1979, production of auxins, cytokinin-like and gibberellin-like substances was proposed for *A. brasilense*, since the increased number of root hairs and of lateral roots observed after inoculation with this bacterium could bemimicked by the application of a mixture of indole- 3–acetic acid, kinetin, and gibberellic acid. Moreover, in several other studies the increased plant growth observed after inoculation with *Azospirillum* was proposed to be due to bacterial phytohormone production (Harari *et al.*, 1988). Eighty per cent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites.

Bacteria belonging to the genera *Azospirillum, Pseudomonas, Xanthomonas, Rhizobium, Alcaligenes, Enterobacter, Acetobacter* and *Bradyrhizobium* have been shown to produce auxins that help in stimulating plant growth (Glick *et al.*, 1998). Isolates producing IAA have stimulatory effect on the plant growth. When the crop is inoculated with the isolates capable of IAA production significantly increases the plant growth by the N, P, K, Ca and Mg uptake of sweet potato cultivar. There is a significant increase in rooting and root dry matter of cuttings of eucalypts when grown on IAA producing rhizobacteria inoculated substrate. Some rhizobacterial isolates stimulates the rhizogenesis and plant growth, maximizing yield of rooted cuttings in clonal nurseries. *Azospirillum brasilense* strain Az39 and *Brayrhizobium japonicum* strain E109 both are able to excrete IAA into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues of Corn (*Zea mays* L) and Soybean (*Glycine max*) and are responsible for their early growth promotion. The use of PGPR isolates is beneficial for rice cultivation as they enhance the growth of rice by inducing IAA production (Ashrafuzzaman *et al.*, 2009).

2.5.2. Synthesis of enzymes that can modulate plant growth and development

Ethylene is a potent plant growth regulator that affects many aspects of plant growth, development and senescence In addition to its recognition as a “ripening hormone”, ethylene promotes adventitious root and root hair formation, stimulates germination, and breaks the dormancy of the seeds. However, if the ethylene concentration remains high after germination, root elongation (as well as symbiotic N_2 fixation in leguminous plants) is inhibited (Jackson, 1991). It is widely believed that many plant growth promoting bacteria may promote plant growth by lowering the levels of ethylene in plants. This is attributed to the activity of the enzyme 1–aminocyclopropane-1–carboxylate deaminase, which hydrolyzes ACC, the immediate biosynthetic precursor of ethylene in plants. The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth (Honma and Shimomura, 1971). In this way the bacterium acts as a sink for ACC and as such is lowering the ethylene level in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations (Glick *et al.*, 1998).

The diazotrophs containing ACC deaminase are present in various soils and offer promise as a bacterial inoculum for improvement of plant growth, particularly under unfavourable environmental conditions such as flooding, heavy metals, phytopathogens, drought and high salt. Inoculation of crops with ACC deaminase-containing PGPR may assist plant growth by alleviating deleterious effects of salt stress. In nature, ACC deaminase has been commonly found in soil bacteria that colonize plant roots (Belimov *et al.*, 2001). Many of these microorganisms are identified by their ability to grow on minimal media containing ACC as its sole nitrogen source. In this way, *Azospirillum* spp., *Herbaspirillum* spp., *Azoarcus, Azorhizobium caulinodans, Gluconacetobacter diazotrophicus, Burkholderia vietnamiensis, Azotobacter* spp., *Azorhizophilus* sp. and *Pseudomonas* spp. are all found to be able to use ACC as the sole nitrogen source for growth.

2.5.3. Antagonistic activity

Rhizobacteria can suppress the growth of various phytopathogens in variety of ways like competing for nutrients and space, limiting available Fe supply through producing siderophores, producing lytic enzymes and antibiosis (Jing *et al.*, 2007). Among PGPRs, fluorescent pseudomonads are widely reported for their broad spectrum antagonistic activity against number of phytopathogens. Han *et al.* (2005) have reported *Delftia tsuruhatensis* strain, HR4, which suppressed the growth of various plant pathogens like *Pyricularia oryzae*, *Rhizoctonia solani* and *Xanthomonas oryzae*. Deliveries of microbial antagonists with urban and agricultural wastes are believed to be the most effective means in suppressing root pathogens of avocado and citrus (Sultana *et al.*, 2006). Recently, different PGPR strains of *Rhizobium meliloti* have been reported to produce siderophores (Arora *et al.*, 2001) in iron stress conditions and thereby added an advantage to exclude the pathogen, *Macrophomina phaseolina*, causing charcoal rot of groundnut. Application of *Pseudomonas aeroginosa* in combination with common medicinal plant *Launaea nudicaulis* also holds good promises for effective control of root infecting fungi of mungbean (Mansoor *et al.*, 2007).

Azospirillum, *Azotobacter*, *Bacillus*, *Enterobacter*, *Paenibacillus*, *Pseudomonas* and *Streptomyces* are recorded as the potent genera of rhizobacteria acting against the pathogens like tomato mottle virus, tobacco necrosis virus, *Rhizoctonia bataticola*, *Myzus persicae*, *Acyrthosiphon kondoi*, *Fusarium avenaceum* etc. Besides, experiments

on the dual effect of PGPR and AM fungi on *Fusarium oxysporum* f. sp., *melongenae* causing brinjal wilt has been made by Kalita *et al.* (2009). PGPR strains such as *Azotobacter* sp., *Azospirillum* sp., and *Pseudomonas fluorescens* and AM fungi like *Glomus fasciculatum*, *G. mossae* and *Gigaspora margarita* are recorded as the most promising microbes to suppress the wilt disease of brinjal, *in vitro*. The microbial inoculants when used as composite inoculum exhibited maximum efficiency in the suppression of diseases with the characteristic increase in chlorophyll content, total number of leaves, shoot height and thereby facilitating overall crop yield than when inoculated singly. However, application of these PGPR strains did not affect populations of beneficial indigenous rhizosphere bacteria including the fluorescent pseudomonads and the siderophore-producing bacterial strains

2.5.4. Mineral solubilzation

One of the various mechanisms by which rhizobacteria promote plant growth is by solubilization of insoluble minerals. Phosphorus is the second most important macronutrient next to nitrogen in limiting crop growth. More than 40% of the world soils are deficient in phosphorus and the acid weathered soils of tropical and sub- tropical regions of the world are particularly prone to phosphorus deficiency (Vance, 2001). A survey of Indian soils revealed that 98 per cent of these need phosphorus fertilization either in the form of chemical or biological fertilizer. Application of chemical phosphatic fertilizers is practised though a majority of the soil P reaction products are only sparingly soluble. Under such conditions, microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. P solubilization by plant-associated bacteria has been well documented in a number of studies. This group covers bacteria, fungi and some actinomycetes. These organisms solubilize the unavailable forms of inorganic-P like tricalcium, iron, aluminum and rock phosphates into soluble forms by release of a variety of organic acids like succinic, citric, malic, fumaric, glyoxalic and gluconic acids (Venkateswarlu *et al*, 2007). Phosphorus solubilizing microorganism include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion. The bacterial genera with this capability are *Pseudomonas*, *Azosirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Flavobacterium* and *Erwinia*

(Richa, 2003). Fixation of atmospheric nitrogen together with solubilization of insoluble phosphate by diazotrophs is rare and to our knowledge such examples of diazotrophic phosphate solubilizer are *Pantoea agglomerans* (Verma *et al.,* 2001) and *Swaminathania salitolerans* (Loganathan and Nair, 2004). Phosphate solubilizing bacteria posses the ability to solubilize insoluble inorganic phosphate by producing acids (Alexander, 1977) rendering it available to plants. However, production of phosphatase in the presence of inorganic sources of phosphorus in the culture media indicated that there could also be a possible role of phosphatase in solubilizing inorganic phosphate. Significantly higher degree of phosphate solubilization from tricalcium phosphate than aluminum phosphate in culture media could be related to the adaptation of the isolates to respective phosphates. The pH of the coastal saline soils in the Sundarbans varies from neutral to slightly alkaline. Such alkaline soils are rich in calcium phosphate, while aluminum phosphate is a major constituent of acidic soils.

Apart from phosphorus, micronutrients like Zn, Fe and Mn are found to be deficient in most of the soils with Zn as a foremost nutrient throughout the world (Alloway, 2001). Zinc, the micronutrient required for plant growth, is an essential component of over 300 enzymes and play catalytic, co-catalytic or structural roles in many plant systems (Christie *et al.,* 2004). For alleviation of Zn and other micronutrients important for crops, their application is done mainly in soluble form as zinc sulphate the soluble form of Zn applied to the soil get transformed into different unavailable forms due to the soil reaction. These transformations are based on the type of soil and other nutrients available. Zn is mainly transformed into zinc carbonate in highly calcareous soils, reacts with Fe and Mn oxide minerals, and while converted into zinc phosphate in higher P fertilizing soils. Inclusion of a bacteria solubilizing zinc, as a bioinoculant in crop production technology is really beneficial for a country like India having high incidence of zinc deficiency (more than 70 per cent). A term called zinc solubilizing bacteria (ZSB) was coined for those bacteria that are capable of solubilizing the insoluble zinc compounds / minerals in agar plate as well as in soil (Anthoni Raj, 2002 and Saravanan *et al.,* 2007). Potassium solubilizing bacteria such as *Bacillus mucilagenosus* and *Bacillus edaphicus* are example of microorganism that used in bio inoculants. Potassium solubilizing bacteria are able to solubilize potassium rock through production

and secretion of organic acids. Potassium solubilizing bacteria is a heterotrophic bacterium which is obtaining all their energy and cellular carbon from pre-existing organic material. Besides, Potassium solubilizing bacteria are aerobic bacteria which play an important role in maintaining soil structure by their contribution in the formation and stabilization of water-stable soil aggregates. In addition, this Gram positive bacterium can produce substance that stimulate plant growth or inhibit root pathogens (Bin Zakaria, 2009). Sheng *et al.* (2008) investigated silicon and potassium mobilization by silicate mineral solubilizing bacteria, *Bacillus globisporus*. In liquid cultures, the strain showed better growth on the biotite than on feldspar and muscovite. The biotite is the best potassium source for growth of the strain and gluconic acid seemed to be the most active agent for the solubilization of the three silicate minerals.

2.5.5. Siderophore and hydrogen cyanide production

In the case of iron uptake, it was suggested that plants can benefit from the siderophores produced by several plant growth promoting rhizobacteria. Although iron is one of the most abundant minerals on Earth, in the soil it is relatively unavailable for direct assimilation by microorganisms. Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Whipps, 2001). Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Many siderophores are non-ribosomal peptides, although several are biosynthesised independently (Raymond *et al.*, 2003; Miethke and Marahiel, 2007). Siderophores are also important for some pathogenic bacteria for their acquisition of iron. Siderophores are amongst the strongest binders to Fe^{3+} known, with enterobactin being one of the strongest of these. Distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups, reveals that most of the isolates belong to Gram negative bacteria corresponding to the *Pseudomonas, Enterobacter* genera, *Bacillus* and *Rhodococcus* genera are the Gram-positive bacteria found to produce siderophores (Tian *et al.*, 2009).

The production of siderophores has been reported for *Azospirillum lipoferum Azospirillum brasilense*, *Azotobacter vinelandii*. Because of the abundance of microbial siderophores in soils, along with their outstanding Fe binding capacity and chemical stability, these compounds may contribute significantly to an increased mobility of Fe in the soil and in the rhizosphere in particular, making it more available for plants. Nevertheless, bacterial siderophores may act as an important source of Fe for higher plants in alkaline and calcareous soils (Bar-Ness *et al.*, 1992), where iron availability is severely limited. There is only one report in which the effect of inoculation with a diazotroph on the iron absorption by plants has been investigated. In this report, *Azospirillum brasilense* was found to increase the iron absorption and translocation by sorghum (Barton *et al.*, 1986).

Cyanide (HCN) production is one of the possible ways by which rhizobacteria may suppress pathogens growth in soil. The plant-parasitic nematodes are among the most destructive plant pests, causing substantial economic losses to agronomic crops worldwide. HCN is potentially an important compound with activity against root knot nematode can act as a useful model system for studying plant-parasitic nematode control using *Pseudomonas* (Aly *et al.*, 2007). Multitrophic interactions mediate the ability of fungal pathogens to cause plant disease and the ability of bacterial antagonists to suppress disease. A pathogen metabolite functions as a negative signal for bacterial antibiotic HCN biosynthesis, which can determine the relative importance of biological control mechanisms available to antagonists and which may also influence fungus-bacterium ecological interactions (Duffy *et al.*, 2004). Positive correlations are found between HCN production in vitro and plant protection in the cucumber/*Pythium ultimum* and tomato/*Fusarium oxysporum* f. sp. *radicis-lycopersici* pathosystems were previously reported. *Bacillus subtilis* is also used as a biocontrol agent. This prevalent inhabitant of soil is widely recognized as a powerful biocontrol agent. In addition, due to its broad host range, its ability to form endospores and produce different biologically active compounds with a broad spectrum of activity, *B. subtilis* as well as other Bacilli are potentially useful as biocontrol agents (Ramette *et al.*, 2003). *B. megaterium* from tea rhizosphere is able to solubilize phosphate, produce IAA, siderophore and antifungal metabolite and thus it helps in the plant growth promotion and reduction of disease intensity (Chakraborty *et al.*, 2006).

2.6. Enhanced stress resistance of diazotrophs

The most problematic environments for diazotrophs are marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and poor water-holding capacity. The long-term goal of improving plant microbe interactions for salinity affected fields and crop productivity can be met with an understanding of the mechanism of osmoadaptation in *Azospirillum* sp. The synthesis and activity of nitrogenases in *A. brasilense* is inhibited by salinity stress. Tripathi *et al.* (2002) reported that in *Azospirillum* sp. there is an accumulation of compatible solutes such as glutamate, proline, glycine betaine and trehalose in response to salinity/osmolarity; proline plays a major role in osmoadaptation through increase in osmotic stress that shifts the dominant osmolyte from glutamate to proline in *A. brasilense*. Saleena *et al.* (2002) have studied the diversity of indigenous *Azospirillum* sp. associated with rice cultivated along the coastline of Tamil Nadu.

Field experiments performed in the 1980s have revealed growth promoting effects of *Azospirillum* on plants exposed to drought stress. Sarig *et al.* (1988) reported that sorghum plants inoculated with *Azospirillum* were less drought stressed, having more water in their foliage, higher leaf water potential, and lower canopy temperature than non-inoculated plants. Total extraction of soil moisture by *Azospirillum* inoculated plants is greater and water is extracted from deeper layers in the soil profile. Therefore, sorghum yield increase in inoculated plants is attributed primarily to improved utilization of soil moisture. Foliar application of a diazotrophic *Klebsiella* sp. could ameliorate drought stress effects on wetland rice, as grain yield increased, together with increased nutrient uptake and proline content. Proline is an important osmoregulator, accumulated as a consequence of drought stress. Creus *et al.* (1997) studied the effects of *A. brasilense* (Sp7) inoculation on water relations in two wheat cultivars. They found that *Azospirillum* stimulated growth of wheat seedlings grown in darkness under osmotic stress, together with a significant decrease in osmotic potential and relative water content at zero turgor. Similarly, in a hydroponic system without nutrients, *A. brasilense* (Sp7) was found to partially reverse the negative effects that drought stress had on wheat seedlings, as it was observed in the growth rate of coleoptiles (Alvarez *et al.*, 1996).

Apart from alleviating osmotic stress in plants, inoculation with diazotrophs can also enhance oxidative stress tolerance. By oxidative stress it is meant the oxidative damage caused by reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Stajner *et al.,* 1995). These highly reactive oxygen species can be generated by the oxidative metabolism of normal cells and by different stress situations. With the production of antioxidant enzymes like superoxide dismutase (SOD), peroxidase, and catalase the cell can neutralize and thus control free radical formation. Also, pigments such as carotenoids could be involved in scavenging singlet oxygen and thus decrease oxidative stress (Elstner *et al.,* 1994). Inoculation with *Azotobacter chroococcum* is reported to improve oxidative stress defense ability in sugar beet leaves since inoculated plants showed increased activities of superoxide dismutase, peroxidase, catalase and increased chlorophyll and carotenoid content (Shan *et al.,* 1992). High activities of antioxidant enzymes (especially SOD) are linked with oxidative stress tolerance. However, the observed effects have not been linked yet to certain traits of the diazotroph. Therefore, it is not clear whether this increase in oxidative stress tolerance is a direct effect of inoculation or rather an indirect consequence of an overall healthier plant by inoculation with *Azotobacter*.

Drought stress causes limitation to the plant growth and productivity of agricultural crops particularly in arid and semi-arid areas. Inoculation of plants with PGPR can enhance the drought tolerance (Figueiredo *et al.*, 2008) that might be due to the production of IAA, cytokinins, antioxidants and ACC deaminase. Inoculation of seeds of *Phragmites australis* with *Pseudomonas asplenii* improved germination and protect the plants from growth inhibition (Bashan *et al.*, 2008). PGPR are also reported as beneficial to the plants like tomatoes and peppers growing on water deficit soils for conferring resistance to water stress conditions (Aroca and Ruiz-Lozano, 2009). More investigations into the mechanisms by which PGPR elicit tolerance to specific stress factors would improve our knowledge on the use of these rhizobacteria in agriculture to provide induced systemic tolerance to water stress.

The application of PGPRs in rhizoremediation technologies is now being considered as effective, since inoculation of PGPR strains could aid remarkable

enhancement in plant growth and development on contaminated Agroclimatic conditions. Rhizobacteria can directly assist rhizoremediation by producing IAA, biological nitrogen fixation, solubilizing phosphorus and secreting siderophores (Denton 2007). PGPR strains, pseudomonads and *Acinetobacter* enhance uptake of Fe, Zn, Mg, Ca, K and P by crop plants (Sonmez *et al.*, 2008). PGPR along with AM fungi are now being utilized in the nutrient poor agricultural soils to increase the solubility of heavy metals and thereby increasing the chances of success in rhizoremediation. Besides, investigations on the application of PGPR strains in decreasing the bioavailability of toxicity resulting in better growth and development in heavy metal contaminated soils through recycling of nutrients, maintaining soil structure, detoxifying chemicals and controlling pests are also well studied (Denton 2007). The metal resistant diazotrophs can serve as an effective metal sequestering and growth-promoting bioinoculant for plants in metal stressed soil. The deleterious effects of heavy metals taken up from the environment on plants can be lessening with the use of plant growth promoting bacteria or mycorrhizal fungi (Rajkumar and Freitas, 2008). The soil microbes, plant growth promoting rhizobacteria, P solubilizing bacteria, mycorrhizal-helping bacteria (MHB) and arbuscular mycorrhizal fungi in the rhizosphere of plants growing on trace metal contaminated soils plays an important role in phytoremediation. Phytoremediation provides a cheap, energy efficient detoxification method that manipulates intrinsic plant characteristics to concentrate the metal contamination in shoot biomass and reduce the bioavailability of the heavy metals. Soil microbes mitigate toxic effects of heavy metals on the plants through secretion of acids, proteins, phytoantibiotics, and other chemicals. Jing *et al.* (2007) reviewed recent advances in effect and significance of rhizobacteria in phytoremediation of heavy metal contaminated soils. Cadmium in soil induces plant-stress ethylene biosynthesis and probably contributes to the accumulation of ACC in roots, the PGPR protect the plants against the inhibitory effects of cadmium. ACC deaminase lowers the ethylene production under cadmium stress condition when measured in vitro ethylene evolution by wheat seedlings treated with ACC deaminase positive isolates (Govindasamy *et al.*, 2009).

Wu *et al.* (2006) carried a greenhouse study with *Brassica juncea* to critically evaluate effects of bacterial inoculation on the uptake of heavy metals from Pb-Zn mine

tailings by plants. The presence of these beneficial bacteria stimulated plant growth and protected the plant from metal toxicity; it had little influence on the metal concentrations in plant tissues, but produced a much larger aboveground biomass and altered metal bioavailability in the soil. As a consequence, higher efficiency of phytoextraction was obtained compared with control treatments. The organism *Pseudomonas putida* is also tolerant to number of heavy metals at higher levels. These characteristics make *P. putida* an excellent candidate for field application in contaminated soil. *P. fluorescens* can survive under dry conditions and hyperosmolarity. On the basis of mutational studies of *Azospirillum*, Kadouri *et al.* (2003) proved the role of PHB synthesis and accumulation in enduring various stresses *viz*., UV irradiation, heat, osmotic pressure, osmotic shock and desiccation. *Azospirillum* inoculated wheat (*T. aestivum*) seedlings subjected to osmotic stress developed significant higher coleoptiles, with higher fresh weight and better water status than non inoculated seedlings.

Numerous studies link the beneficial effects of inoculation with ACC deaminase producing endophytic bacteria with increased stress tolerance and growth in suboptimal conditions. The inoculation of tomato, cotton, groundnut, canola, maize and wheat with the ACC deaminase producing bacteria *Achromobacter piechaudii* AVR8, *Klebsiella oxytoca* RS-5, *Serratia proteamaculans* M35, *Enterobacter cloacae* CAL2 and *Pseudomonas* spp. increased host biomass production, lowered Na^+ and enhanced K^+ cell content compared to uninoculated plants (Newman and Reynolds, 2005). *Burkholderia phytofirmans* PsJN is an intensively studied endophyte that has been associated with growth promotion and enhanced stress tolerance in several plant species, including potato, vegetables and grapevine (Sessitsch *et al.*, 2002). *B. phytofirmans* PsJN has ACC deaminase activity and the plant growth enhancement under environmental stress has been postulated to be associated with ACC deaminase production by the bacterium. The enhanced growth was associated with an increase in plant photosynthetic capacity and starch content, as well as proline and phenolic contents in plant cells. This indicated enhanced cold tolerance of plants by PsJN inoculation (Aroca and Ruiz-Lozano, 2009). Inoculation of the alpine plant species *Chorispora bungeaana* with endophytic *Clavibacter* sp. Enf12 isolated from the same plant growing under snow enhanced plant growth both at 20ºC and 0ºC. It also significantly attenuated the

production of ROS, oxidative damage and electrolyte leakage. Inoculation also led to elevated levels of antioxidant enzymes and proline, indicating improved control of oxidative damage and increased hardiness (Desbrosses *et al.*, 2009).

2.7. Effect of plant growth promoting diazotrophs on growth and yield of response of cereals

Seed bacterization with plant growth promoting rhizobacteria has emerged as a promising technique to induce enhanced growth of plants and simultaneously provide protection from deleterious and pathogenic micro organisms. Plants play an important role in selecting and enriching the type of bacteria by the constituents of their root exudates. The bacterial community developing in the rhizosphere has an efficient system for uptake and catabolism of organic compounds present in root exudates (Barraquio *et al.*, 1997). Rice is one of the major staple food crops of the world's population. Germinated rice seeds when inoculated with *Azospirillum* appeared to be taller after 30-35 days when compared with non-inoculated controls. *Burkholderia vietnamiensis* when used to inoculate rice in field trial, it increased grain yield significantly upto 5 per cent, shoot weight 33 per cent, root weight 57 per cent, leaf area 30 per cent and number of tillers 13 per cent (Tran van *et al.*, 2000). Several studies indicate that diazotrophs has the ability to improve plant growth and yield of crop. In case of wheat, PGPR inoculation enhances the rate of seedling emergence, tiller formation and plant dry weight. *Azorhizobium caulinodans* has been found to increase the growth, dry weight and nitrogen content of wheat plant (Matthews *et al.*, 2001). It has been reported that seed inoculation with various *Azotobacter* cultures increases the grain yield by 38.5 per cent, straw yield by 15.3 per cent, number of tillers by 12.5 per cent, spikelets by 10.7 per cent and 1000 grain weight by 7.3 per cent.

Pedraza *et al.* (2009) assessed the *Azospirillum* inoculation and N-fertilization effect on grain yield and on the phyllosphere endophytic diversity of nitrogen-fixing bacteria in a rice rainfed crop. Authors used cultivation-based techniques and cultivation-independent methods involving PCR 16S rRNA and denaturing gradient gel electrophoresis (DGGE). Results showed that grain yield was improved when inoculated with *Azospirillum* (depending on the genotype) and/or fertilized with urea. A similar

behavior was observed in total N-content in grain and the MPN determination, as the highest values occurred when seeds were inoculated with *A. brasilense* REC3 (S1) than with *A brasilense* 13-2C (S2). A positive nitrogenase activity and PCR-*nifH* amplification suggests that the bacteria associated to inner tissues of rice phyllosphere could have contributed to the different N-contents detected. Application of *A. brasilense* strains as inoculants did not influence the dominant members of the endophytic microbial communities in the phyllosphere, but improved N content and production of rainfed rice crop.

Many members of genera *Pseudomonas* perform beneficial effects on plants (Lifshitz, 1987). Among them *P. putida* and *P.fluorescens* are dominant group (Haas and Defago, 2005). Diazotrophic *Pseudomonads* are commonly isolated from the rhizosphere of wetland rice (Barraquio and Watanabe, 1983). De Freitas *et al.* (1997) observed the enhanced growth of wheat plants inoculated with rhizobacteria *Pseudomonas fluorescens*. Kloepper *et al.* (1980) reported that several strains of *Pseudomonas fluorescens* when applied to seeds increased the plant growth of rice and cotton by 27 and 40 % respectively. Mirza *et al.* (2006) reported that the effect of nitrogen fixing *Pseudomonas* strain K1 on rice grain yield was comparable to those of *A. brasilense* Wb3 and *Zoogloea* sp. Ky1 for rice varieties such as Super Basmati and Basmati 38. Inoculation of *Pseudomonas* strain increased rice yield by 55 % and 93% per cent respectively for Super Basmati and Basmati 38. These results show that nitrogen-fixing Pseudomonads deserve attention as potential PGPR inoculants for rice.

In rice, seed treatment with four different diazotrophic bacteria *viz*., *Azospirillum brasilense* (Sp7), *Herbaspirillum seropedicae* (Z78), *Enterobacter* sp. (L2) and *Gluconacetobacter* sp. showed the better plant growth with the ability of N fixation, produces phytohormone indole-3-acetic acid in different concentration (Keyeo *et al.*, 2011).

Inoculation of *Sphingomonas* sp. RFNB22 remarkably increased plant height and dry biomass production compared with the control. However, the nitrogenase activity of pure cultures of the strains did not correlate much with their performance in inoculated rice plants was reported by Kim *et al.* (2005). Several reports revealed that inoculation with free living diazotrophs like *Azotobacter*, *Pseudomonas* and *Azospirillum* increased

the yield of rice by 20–55% (Yanni and El- Fattah 1999 and Balandreau, 2002) and a strain of diazotrophic *Burkholderia* increased the rice plant biomass by 69% (Kennedy *et al.*, 2004). Furthermore, inoculation with diazotrophic bacteria increased the level of N accumulation in 45-day-old rice seedlings, which reached 20.2% in the treatment with *Herbaspirillum* (Mirza *et al.*, 2006).

Baldani *et al.* (2000) reported that ^{15}N labelled strains of endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* inoculated rice seedlings showed the increase in N content. Govindarajan *et al.* (2008) studied the effects of the Inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. Furthermore authors observed that higher nitrogen fixation in Rice (ADT-43) where nearly 42% (field) and 40% (pot) of the nitrogen was derived from the atmosphere (% Ndfa). Phan *et al.* (2009) studied the inoculation effect of plant growth-promoting microorganisms enhance utilisation of urea-N and grain yield of paddy rice in southern Vietnam.The results indicated that inoculation with BioGro, containing a *Pseudomonas*, two bacilli and a soil yeast, significantly increased grain and straw yields and total N uptake in both seasons, as well as grain quality in terms of percentage N.

Biswas *et al.* (2000) reported the increased mineral uptake by plants has been suggested to be due to a general increase in the volume of the root system, as reflected by an increased root number, thickness and length, and not to any specific enhancement of the normal ion uptake mechanism Higher K and Fe uptake for instance are related to thicker roots and higher P uptake to the presence of root hairs. On the other hand, experiments with *Azospirillum* species have suggested that this organism specifically enhances mineral uptake (Murty and Ladha, 1988). It has been demonstrated that *Azospirillum*-inoculated plants take up minerals (N, P, and K) from solutions at faster rates than uninoculated controls and, consequently, plants in the field accumulate dry matter, N, P, and K, at higher rates.

PGPR can change the plant physiology and certain nutritional and physical properties of rhizospheric soil and indirectly influence on the colonization patterns of soil microorganisms in that particular region. Inoculation of rhizobacteria increased uptake of nutrient elements like Ca, K, Fe, Cu, Mn and Zn by plants through stimulation of proton

pump ATPase (Mantelin and Touraine, 2004). Reports are available on the combinations of *Bacillus* and *Microbacterium* inoculants to improve the uptake of the mineral elements by crop plants (Karlidag *et al.*, 2007). This increase in nutrient uptake by plants might be explained through organic acid production by the plants and PGPRs, decreasing the soil pH in rhizosphere. Ample evidences (Glass *et al.*, 2002) are there on the maintenance of soil fertility by the rhizobacterial isolates to increase the availability of nutrients for plants. Solubilization of unavailable forms of nutrients is one of the essential criteria in facilitating the transport of most of these nutrients (Glick, 1995).

This review recognizes the role of biological nitrogen fixation as a non-polluting and more cost-effective way to improve soil fertility. A wide diversity of diazotrophic species both symbiotic and free living association with nonlegumes are found superior to other systems with respect to N_2 fixing potential and adaptation to severe conditions. These associations might have sufficient traits necessary to establish successful growth and nitrogen fixation under the conditions prevailing in non-leguminous cropping system of India.

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The present investigation was carried out to study the culturable diazotrophic bacterial diversity of selected grass species which are naturally grown in various physiographic regions. These diazotrophs were screened for nitrogen fixation and identified based on 16S rRNA gene sequencing. The multiple growth promoting activities and the tolerance to various abiotic stresses were also analyzed. Influence of diazotrophic bacterial inoculation on the growth of rice was studied under *in vitro* conditions as well as under field conditions. The details of the materials employed and methods followed during the course of investigation are described in this chapter.

3.1. General

3.1.1. Location

All the laboratory experiments were conducted in the Department of Agricultural Microbiology, Directorate of Natural Resource Management, Tamil Nadu Agricultural University, Coimbatore, situated at an altitude of 426.7 m above mean sea level, 11°N latitude and 77°E longitude. Field experiment was conducted in Wetland Farm, Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore.

3.1.2. Agroclimatic conditions

The mean minimum and maximum temperature during the investigation period at Coimbatore were 20°C and 34.2ºC, respectively. The mean relative humidity was 63.5 per cent and the average annual rainfall was 670 mm.

3.1.3. Glassware

The glasswares of Borosil grade were used in all the experiments. Glasswares were cleaned with soap solution, soaked in chromic acid solution (100 g potassium dichromate dissolved in one litre of water with 500 ml of concentrated sulphuric acid) for 2 h, rinsed twice with distilled water, dried and sterilized in an hot air oven at 180°C for 3 h before use.

3.1.4. Chemicals

All the chemicals used for the study were of Analytical Reagent (AR) grade obtained from M/S. Hi-media, Qualigens, Merck and Sigma Chemicals.

3.1.5. Enzymes and markers

All enzymes and primers used in molecular analysis were obtained from M/s. Merck (Pvt.) Ltd, Bangalore and M/S. Sigma Aldrich, USA.

3.1.6. Composition of media, reagents, buffers and solutions

The composition of different media used and reagents are given in Annexure II. The solutions and buffers used for molecular studies are given in Annexure III.

3.1.7. Sterilization techniques

The glasswares were sterilized in an hot air oven (Lab Companion, Korea) at 180°C for 3 h. All growth media, broth and physiological salines were sterilized in an autoclave at 15 lbs pressure for 20 min in autoclave (Tomy, Japan). Vitamin solutions, growth hormones and antibiotics were filter sterilized using 0.22 μm nitrocellulose membrane filter (Sartorius, US). Isolation, purification, inoculation and other microbiological works were carried out in a laminar air flow chamber (Yorco Horizontal, New Delhi).

3.1.8. Standard cultures

1 For regular experiments, standard cultures of *Azospirillum lipoferum* (Az 204) and *Gluconacetobacter diazotrophicus* (PAL5) maintained at the Bioinoculant Production and Quality Control Laboratory, Department of Agricultural Microbiology, TNAU, Coimbatore-3 were used.

2 The plant growth promoting rhizobacterial (PGPR) strain, *Pseudomonas fluorescens* (Pf1) was obtained from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatote-3, India.

3 For molecular studies, *E. coli* DH5α maintaining at Department of Agricultural Microbiology, TNAU, Coimbatore-3 was used for the present study.

3.1.9. Seed materials

Rice seeds (variety. ADT 43) obtained from the Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore were used for the gnotobiotic and field study.

3.1.10. Replications

All the *in vitro* and *in vivo* experiments were replicated thrice unless and otherwise specified.

3.2. Sampling

Soil and plant samples were collected from different physiographic regions experiencing various abiotic stresses. Based on their predominance in each region, a total of 10 different grass species (*Brachiaria reptans*, *Cenchrus glaucus*, *Saccharum spontaneum*, *Panicum repens*, *Cyperus rotundus*, *Dactyloctenium aegyptium*, *Chloris barbata*, *Oryza rufipogon*, *Cyanodon dactylon* and *Setaria verticillata*) were sampled (Table 3). Plants were uprooted carefully and the soil adhering to the root was separated in a sterile petri dish and mixed thoroughly so as to make a composite sample for microbiological analysis. Plant samples and soil samples collected were transported to laboratory in ice box for further analysis. The samples were used immediately for preliminary analyses and stored at 4°C in a refrigerator for further studies (Pramer and Schmidt, 1966).

3.3. Physico-chemical and biological properties of soil collected from different physiographic regions

3.3.1. Methods employed for physico-chemical analyses of soil

Soil property	Method	Reference
pH (Soil: Water = 1: 2)	glass electrode in the Cyber Scan 510	Jackson (1973)
Electrical conductivity (dS m^{-1}) (Soil: Water = 1: 2)	ELICO conductivity bridge	Jackson (1973)
Exchangeable sodium percentage (ESP %)	$\frac{Na^{+}}{Na^{+}+Ca^{++}+Mg^{++}+K^{+}} \times 100$	Saxena *et al.* (1978)
Available N (kg ha^{-1})	Alkaline permanganate method	Subbiah and Asija (1956)
Available P (kg ha^{-1})	0.5 M $NaHCO_3$ of pH 8.5 extract by spectrophotometry	Olsen *et al.* (1954)
Available K (kg ha^{-1})	Flame photometric method using neutral normal ammonium acetate extract	Stanford and English (1949)

Table 3. Details of Grass species from different physiographical regions of India used for the present investigation

Grass species	Sampling site	Latitude	Longitude	Physiographic region
Brachiaria reptans (Water grass)	Barrackpur, Kolkata,West Bengal	88° 34´ 5.1” E	22° 19´ 49.6” N	Indo Gangetic alluvial plain
Cenchrus glaucus (Buffel grass)	Chadrapur Ganjam,Orissa	88° 24´ 22.8” E	19° 24´ 21.09” N	Eastern Ghats
Saccharum spontaneum (Wild sugarcane)	Madan Mahal, Jabalpur, Madhya Pradesh	79° 40´ 50.33” E	22° 51´ 17.03” N	Central highlands
Panicum repens (Torpedo Grass)	Maruteru, West Godaveri, Andrapradesh	80° 59´ 38.86” E	16° 30´ 39.7” N	Deccan Plateau
Cyperus rotundus (Nut grass)	Chickarasinikere, Mandya, Karnataka	77° 3´ 35.9” E	12° 17´ 34.78” N	Reverain land form
Dactyloctenium aegyptium (Crowfoot grass)	Kasargod, Kerala	75° 7´ 59.81” E	12° 24´ 31.4” N	Kerala plains
Chloris barbata (Finger grass)	Thavalakuppam, Pudhucherry	76° 46´ 54.7” E	11° 23´ 12.6” N	Coastal plains
Oryza rufipogon (Wild rice)	Gudalur, Ooty,Tamil Nadu	79° 51´ 33.1” E	11° 54´ 32.52” N	Western Ghats
Cyanodon dactylon (Bermuda grass)	Navalur kutapattu, Trichy,TamilNadu	79° 46´ 34.9” E	10° 33´ 21.32” N	Reverain land form
Setaria verticillata (Bristly foxtail)	Thirupoondi, Nagapattinam, Tamil Nadu	79° 53´ 37.6” E	10° 46´ 25.67” N	Coastal plains

3.3.2. Enumeration of microbial population

Soil samples obtained from various physiographic regions were analyzed for enumeration of total bacteria, fungi, actinobacteria and diazotrophs. Enumeration was done by employing serial dilution and plating technique with appropriate media. Tenfold serial dilutions were prepared for each soil sample till appropriate dilution was obtained.

3.3.2.1. Enumeration of bacterial population (James, 1958)

Enumeration of bacteria was carried out in soil extract agar medium (Annexure II) using standard dilution plating technique. One g of soil sample was serially diluted by 10 fold series using sterile physiological saline upto 10^{-7} and one ml of aliquot from 10^{-7} dilution was taken and dispensed in sterile petriplates with soil extract agar medium. The bacterial colonies were enumerated after 48 h of incubation (Lab Companion, Korea) at 37°C and expressed as number of colony forming units (cfu) g^{-1} dry weight of soil.

3.3.2.2. Enumeration of fungal population (Parkinson *et al.,* 1971)

Enumeration of fungi was carried out in Martin Rose Bengal Agar medium (Annexure II). One g of soil sample was serially diluted by 10 fold series using sterile physiological saline up to 10^{-4} and one ml of aliquot from 10^{-4} dilution was taken and dispensed in sterile petriplates with Martins Rose Bengal Agar medium. The fungal colonies were enumerated after 5 days of incubation (Lab Companion, Korea) at 37°C and expressed as number of colony forming units (cfu) g^{-1} dry weight of soil.

3.3.2.3. Enumeration of actinobacterial population (Wellingtonn and Toth, 1963)

Enumeration of actinobacteria was carried out in Kenknight's Agar medium (Annexure II). One g of soil sample was serially diluted by 10 fold series using sterile physiological saline up to 10^{-3} and one ml of aliquot from 10^{-3} dilution was taken and dispensed in sterile petriplates with Kenknight's Agar medium. The actinobacteria colonies were enumerated after 7 days of incubation (Lab Companion, Korea) at 37°C and expressed as number of colony forming units (cfu) g^{-1} dry weight of soil.

3.3.2.4. Enumeration of diazotrophic population (Döbereiner, 1989)

Enumeration of diazotrophs was carried out in modified Nfb medium (Annexure II)**.** One g of soil sample was taken and 10 fold dilution series was prepared up to 10^{-4}, one ml of aliquots from 10^{-4} dilution was taken and dispensed in sterile Petriplates with modified Nfb medium. The diazotroph colonies were enumerated after 5 days of incubation (Lab Companion, Korea) and expressed as number of colony forming units (cfu) g^{-1} dry weight of soil.

3.3.3. Soil enzyme activity

3.3.3.1. Fluorescein diacetate (FDA) hydrolysis (Schnurer and Rosswall, 1982)

The determination of FDA hydrolysis was carried out in 2 g of field moist soil, where 15 ml of 60 mM potassium phosphate buffer (pH 7.6) was added. 100 µl of substrate solution was added to samples. After 20 min of incubation, the reaction was stopped by adding 15 ml of chloroform: methanol (2:1). After filtering the solutions (Whatman No. 42), absorbance was measured at 490 nm on a UV Visible Spectrophotometer (Cary 50 BIO, Varian) and calculated from the standargraph of fluorescein. The FDA hydrolysis rate was expressed as µg flurescein g^{-1} h^{-1}.

3.3.3.2. Arginine deaminase (ADA) activity (Alef and Kleiner, 1986)

The determination of ADA activity was measured in wet soil samples (40% WHC), where 0.2% arginine solution was added and then the samples were kept for 3 hours in dark at 25°C. Then, the samples were frozen at –15°C during 24 h, in order to stop ammonification process. After incubation, samples were de-frozen; the ammoniacal nitrogen in samples was determined by the salicylate method (Kempers and Zweers, 1986). In this method the aliquots of 2.5 ml from the sample were added to 1.25 ml of Na-nitroprussside solution and vortexed briefly for few seconds. To this 0.5 ml of dichloroisocynaourate was added and the samples were incubated for 30 min in dark. The absorbance of the sample was measured at 625 nm on a UV Visible Spectrophotometer (Cary 50 Bio, Varian) and calculated from the standard graph with ammonium chloride. ADA was expressed as µg g^{-1} soil h^{-1}.

3.4. Isolation of diazotrophs from rhizosphere soils of selected grass species

Diazotrophic microorganisms were isolated using serial dilution technique on four selective N-free media *viz*., NFMM (Piao *et al*., 2005), LGI-P (Reis *et al*., 2000), TDM (Döbereiner, 1989) and JNFb (Kirchhof *et al*., 1997) (Annexure II). Aliquots (0.1 ml) from the serially diluted samples (10^{-3} to 10^{-6}) were added to four different media in Petri plates and kept in an incubator at 30°C. Five days after incubation, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies were picked from the Petri dishes and sub-cultured to obtain pure cultures. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at –80°C.

3.5. Isolation of diazotrophs from plant tissues of selected grass species

Plants were carefully removed from soil by repeated washing and separated into stems and roots. The stems and roots were cleaned again thoroughly with tap water and deionized water and drained on absorbent towels. All tissues were surface sterilized as described by Stoltzfus *et al*. (1997). Ten grams of tissue was shaken for thirty minutes in 500 ml Erlenmeyer flask containing 250 ml sterile deionized water. The tissue was washed with 70% ethanol for 30 seconds, washed with fresh sodium hypochlorite solution (2.5% available chlorine) for 5 min and then sterilized with 0.1% $HgCl_2$ for 3 min for roots, 5 min for stems. The tissue was then washed ten times with sterile water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptic soy agar (TSA) medium plates. The plates were examined for bacterial growth after incubation (Lab Companion, Korea) at 28°C for 3 days.

Specimen of washed roots, stems and leaves were macerated and 0.1 ml of serial dilutions in 4% (commercial) sugar solution up to 10^{-6} and there dilutions were inoculated into vials containing selective N-free semi solid media *viz*., NFb **(**Döbereiner, 1989), JNFb (Kirchhof *et al*., 1997), SM (Reinhold *et al*., 1985), SSM (Reinhold *et al*., 1986), Rennie (Rennie, 1980), LGI (Reis *et al*., 2000) (Annexure-II). After four to six days of incubation at 30°C, the population size was estimated by the MPN method and pellicle forming bacteria were subjected to further purification by streaking on N free agar plates. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at –80°C.

3.5.1. Authentication of putative endophytes

To authenticate the endophytes from non endophytes, re-isolation step from inoculated plants under controlled conditions was done. Rice seeds (variety ADT 43) were gently dehulled, placed in 70% ethanol for five minutes, washed with sterile distilled water, sterilized by addition of 0.2% $HgCl_2$ for 4 minutes and washed 6 times in sterile distilled water. Seeds were placed on plates containing TY medium medium and incubated at 30°C for two days to allow germination and check for contamination. Seeds showing no contamination were placed in culture tubes containing 50 ml of modified Fahraeus medium (Fahraeus, 1957).

Bacteria were re-isolated from the inoculated seedlings as follows. Seedlings were grown for 21 days and carefully removed from the growth tubes. The roots and shoots were separated and sectioned to small pieces. The tissues were placed in 100 ml Erlenmeyer flasks containing 10 ml of sterile distilled water, shaken for 30 minutes and washed with 10 ml sterile distilled water. The tissue was sterilized by adding 10 ml of 0.2% $HgCI_2$ for 30 seconds and washed six times with sterile distilled water. Tissues were macerated in one ml sterile distilled water and 20 µl of macerate was spread onto plates containing TY medium. The colonies from plates were purified and used for further analysis.

3.5.2. Biochemical activities of the endophytes

3.5.2.1. Cellulase activity test (Rautela and Cowling, 1966)

The test isolates were spot-inoculated on the swollen cellulose agar plates and incubated for one week at 30°C. After incubation, the clear zone around the colonies after flooding the plates with 1% aqeous hexadecyl trimehtyl-ammonium bromide was observed.

3.5.2.2. Pectinase activity test (Cotty *et al*., 1990)

The test isolates were spot-inoculated on the pectin agar plates and incubated for one week at 30°C. The plates then were flooded with 0.1% aqueous Red ruthenium solution for one hour, drained, rinsed with water and observed. Red ruthenium is bound to unhydrolysed pectin and gives the red color. Halo zone around isolate's colony was observed.

3.5.2.3. Motility test (Elbeltagy *et al*., 2000)

Each isolate was spot-inoculated on the centre of soft agar plates (0.2% agar) and incubated at 30^{o}C. The diffusion of colony was observed and recorded at 24 h.

3.6 Preliminary screening for diazotrophy of the isolates obtained from selected grass species

3.6.1 Growth in N-free medium (Burris and Wilson, 1972)

The bacterial isolates were grown in Nfb broth without addition of NH_4Cl as a unique nitrogen source were incubated at 28°C for 7 days and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation. The growth measurement was taken in a UV- VIS spectrophotometer (Cary 50 Bio, Varian) at 660 nm and expressed as - no growth (< 0.10 OD) ; + (0.1 to 0.3); ++ (0.3 to 0.5); +++ (>0.5).

3.6.2. Estimation of total Nitrogen (Humphries, 1956)

Nitrogen free liquid malate medium was prepared dispensed into 100ml quantities in 250ml Erlenmeyer flasks and sterilized. One ml of inoculum (10^7 cells ml^{-1}) was added to each flask and incubated at 32^{o}C under static conditions. After 7 days, 5 ml quantities of the homogenized broth was withdrawn and digested with 5 ml quantities of sulphuric acid, one drop of perchloric acid was added at the end and the digestion was continued till the solutions turned as clean and colourless. After cooling, the contents were transferred to a microkjeldahl distillation unit with the addition of 10 ml of 40 per cent sodium hydroxide. Ammonia evolved was collected over 2 per cent boric acid (20 ml) containing a drop of double indicator (83.3 mg of bromocresol green and 16.6 mg of methyl red dissolved in 10 ml of 95 per cent ethyl alcohol) which was back titrated against 1/500 N sulphuric acid (0.000028 g of N). The quantity of nitrogen content in the medium was expressed as mg g^{-1} of malate.

3.6.3. Estimation of ammonia excretion (Cappuccino and Sherman, 1992)

Bacterial isolates were tested for the excretion of ammonia in peptone water. Freshly grown cultures of were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 30^{o}C. Nessler's reagent (0.5 ml) was added in each tube. Reaction was let to proceed for at least 10 min after adding Nessler's reagent. Colour was

measured in sample by using UV VIS spectrophotometer (Cary 50 Bio, Varian). The reddish brown hue typical of ammonia nitrogen was measured in the wavelength of 400 nm. The concentration of ammonia present in the sample was determined from standard curve prepared using ammonia solution and expressed as mg ml^{-1}.

3.7. Acetylene Reduction Assay (Bergersen, 1980)

The nitrogen fixing capacity of the diazotrophic cultures was evaluated by estimating the acetylene reduction assay using Gas chromotography. Twenty five ml of modified N-free malate broth and semisolid Rennie medium were prepared in 100 ml vials for rhizosphere and endophytic isolates respectively. The vials were inoculated with the cultures and were incubated under static condition in an incubator at 28 ± 2°C. After 5 days of growth, the cotton plugs were replaced by rubber septa and capped with aluminium caps. The head space volume was replaced by 5% (v/v) acetylene which was generated from calcium carbide and water. The culture was incubated for 24 h. After incubation, 1 ml of gas sample was withdrawn and injected into the Gas chromatograph (Chemito-7610) equipped with FID detector and Porapak N column (6" x ⅛") . Temperatures of injector, detector and column were maintained at 110°C, 120°C and 73°C respectively. Nitrogen gas was used as carrier gas at the flow rate of 30 ml min^{-1}. The bacterial cells in the medium were evenly mixed and the protein concentration was measured (Bradford, 1976).

3.8. Carbon substrate utilization pattern of diazotrophs from grass species

All the diazotrophic isolates were analyzed for growth in different carbon substrate *viz.*, D-glucose, D-xylose, amylose, fructose, mannitol, betaine, citrate, cellulose, starch, glycerol, tartrate and acetate. These carbon substrates were substituted for malate in Nitrogen free liquid medium (Baldani and Döbereiner, 1980) at 1 per cent (w/v) level. Presence of growth was observed after 15 days of incubation at 30ºC and growth was compared to a negative control containing no added carbon source. Carbon utilization pattern of the isolates was grouped based on the Jaccard's similarity coefficient. In order to determine the similarity between the isolates, a binary matrix was established recording the presence or absence of growth in respective carbon substrate substituted medium. UPGMA algorithm was used for hierarchical cluster analysis.

Pair-wise comparisons were calculated using Jaccard's coefficient (Jaccard, 1912) and dendrogram was built using the UPGMA method (Nei and Li, 1979) using NTSYS-PC2 package (Numerical taxonomy analysis program package, External software, USA).

3.9. Intrinsic antibiotic resistance (IAR) profile of diazotrophs from grass species

Thirty two diazotrophic isolates were screened for resistance to a panel of thirty six antibiotics by the disc diffusion method. Ready to use antibiotic discs (Hi-media Dodeca Universal DEO 008) containing 12 antibiotic discs of 0.6 mm diameter were used. The antibiotics used were representatives of the various classes of antimicrobial agents in common use (Annexure II).

Hundred µl of log phase culture was spread evenly on solidified LB medium and the surface was allowed to dry for few min. The antibiotic dodeca disc was placed on the surface and the Petriplates were incubated at 28 ± 2°C for 48 h. The susceptibility of the isolate was indicated by development of clearing zone around each disc. Antibiotic susceptibility profile of the isolates was grouped based on the Jaccard's similarity coefficient. UPGMA algorithm was used for hierarchical cluster analysis. Pair-wise comparisons were calculated using Jaccard's coefficient (Jaccard, 1912) and dendrogram was built using the UPGMA method (Nei and Li, 1979) using NTSYS-PC2 package (Numerical taxonomy analysis program package, External software, USA).

3.10. Authentication of diazotrophs obtained from various grass species by 16S rRNA gene sequencing

3.10.1. Isolation of total genomic DNA

The genomic DNA from 24 selected isolates, which showed positive for acetylene reduction activity, was extracted using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method as given by Melody (1997) with minor modifications. Overnight grown culture of 25 ml quantity was centrifuged at 6,000 rpm for 5 min at 4°C. The supernatant was discarded, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells, followed by centrifugation at 5000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of

butanol. The pellet was resuspended in 1 ml TE buffer added with 100 µl lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10 per cent SDS and 25 µl of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for 1 h. To this, 200 µl of 5 M NaCl was added and mixed well. CTAB (10 per cent CTAB in 4.1 per cent NaCl solution) (150 µl) was added, mixed well and incubated at 65°C for 10 min. The extract was deproteinized by 1 ml of phenol: chloroform mixture and centrifuged at 6000 rpm for 15 min at 4°C. The aqueous layer was transferred carefully to a new 2 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice- cold isopropanol, incubated for 1 h to overnight at -20°C. The DNA was pelletized by centrifugation at 12000 rpm for 15 min at 4°C and the pellet was washed with 70 per cent ethanol, dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One µl of DNAse free RNAse (10 mg ml^{-1}) was added by swirling and incubated at 37°C for 30 min. The DNA was stored at -20^{o}C for further use.

3.10.2. 16S rRNA gene amplification

Full-length 16S rRNA gene (1500 bp) was amplified from the isolates by PCR using the universal eubacterial forward primer fd1 (5' AGA GTT TGA TCG TGG CTC AG 3') and the reverse primer rp2 (5' ACG GCT ACC TTG TTA CCA CTT 3') (Weisburg *et al*., 1991). The 45 µl PCR reaction mixture consisted of DNA template 50 ng, 1X Taq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 2.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase (M/S Merck, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) with following conditions: initial denaturation at 95°C for 1 min, 35 cycles consisting of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min. The amplified products were analysed by electrophoresis in 1.5 per cent agarose gels. The gels were viewed and photographed using InGenius (Syngene, UK) gel documentation and analysis system.

The band of the expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and eluted using sterile milli-Q water.

3.10.3. Cloning using T/A cloning vector in *E. coli*

The gel- purified PCR product of 16S rRNA gene of each isolate was cloned in to T/A cloning vector, pGEM®-T (Promega, USA) according to the manufacturer's instruction. The 12µl of ligation mixture contains 6 µl of 2X T4 DNA ligase buffer, 4.25 µl of PCR product, 0.75 µl of pGEM®-T vector, 1 µl of T4 DNA ligase and 4 µl of water, mixed gently without pipetting and incubated at 16°C for overnight. The quantity of 10 µl of ligation mixture was used for transformation in *E. coli* DH5α competent cells. The transformation was performed by following $CaCl_2$ mediated transformation using heat- shock at 42°C for 60 sec. The transformants, with insert displayed as white colonies in LB agar plate supplemented with Ampicillin (100 µg ml^{-1}); X gal (20 mg ml^{-1}) and IPTG (100 mM). The presence of insert was reconfirmed by colony- PCR using M13 forward and reverse primers (Fermentas, USA). In 20 µl of PCR mixture, (same composition as 16S rRNA gene with M13 F and M13 R primers), a loopful of colony was transferred using sterile toothpick and PCR was performed in thermocycler (Eppendorf Master cycler, Germany) with the programme same as described for 16S rRNA gene.

The band of expected size (about 1700 bp) was visualized and photographed using InGenius (Syngene, UK) documentation and analysis system.

3.10.4. Sequencing

Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

3.10.5. Phylogenetic analysis

The identity of 16S rRNA gene sequences was performed by similarity search using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The closest species, strain and per cent similarity to the isolates were obtained from the BLAST result.

The phylogenetic tree was constructed with existing 16S rRNA gene sequences from closely related bacteria, obtained from NCBI GenBank database. The phylogenetic tree was constructed by neighbor-joining method of Saitou and Nei (1987) using MEGA 5.0 software (Tamura *et al*., 2011).

3.10.6. Amplification of *nifH*

The genomic DNA of the isolates extracted and stored at ⁻20°C was used as template. The forward primer nifHb1 (5' GGC TGC GAT CCC AAG GCT GA 3') (Burgmann *et al*., 2004) and reverse primer CDHPnif723R (5' GAT GTT CGC GCG GCA CGA ADT 3') (Steward *et al*., 2004) were used for amplification of partial *nifH* gene (450 bp) (Buckley *et al*., 2007).

The 45 µl PCR reaction mixture contained 50 ng of DNA template, 1XTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 2.5 mM $MgCl_2$ and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) using conditions: initial denaturation at 95°C for 5 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 5 min. The amplified products were analysed by electrophoresis in 1.5 per cent agarose gels. After separation of the PCR products in agarose gel, it was viewed and photographed using InGenius (Syngene, UK) gel documentation and analysis system.

3.10.7. ARDRA

Approximately 1 µg of PCR-amplified 16S rRNA gene fragments were restricted with endonuclease *Hae*III (Fermentas, USA) at 37°C for 3h and resolved by electrophoresis in 2% metaphor agarose gels. Banding patterns were visualized by ethidium bromide staining and documented in InGenius (Syngene, UK) documentation and analysis system.

3.11. Plant growth promoting activities of diazotrophic isolates from grass species

3.11.1. Indole Acetic Acid (IAA) production (Chandramohan and Mahadevan, 1968)

One ml of the cultures at exponential stage was inoculated in 100 ml LB medium containing filter sterilized L-tryptophan (0.01per cent w/v). All the flasks were wrapped with black paper to avoid photo inactivation of the biologically active compounds. The flasks were incubated at room temperature for 7 days. The cells were harvested by centrifugation at 10,000 rpm for 5 min and the supernatant was collected and concentrated to 25 ml. The culture filtrate was adjusted to pH 2.8 with 1N HCl and equal

volume of ice cold (4°C) diethyl ether was added. The contents were shaken well and allowed to stand in dark for 4 h with intermittent shaking. Using a separating funnel, the aqueous phase was separated from organic phase and the extraction was repeated 3 times. Discarding the aqueous phase, the organic phases were pooled and evaporated to dryness in the dark. The residue was dissolved in 2 ml of absolute methanol.

The quantity of IAA produced was estimated using Salper's reagent (1 ml of 0.5N $FeCl_3$ mixed in 50 ml of 35per cent perchloric acid) (Gordon and Paleg, 1957). A quantity of 0.5 ml methanol fraction was added to 1.5 ml of distilled water and 4.0 ml of Salper's reagent and incubated in darkness for 1 h at 28°C. The intensity of pink colour developed was read in spectrophotometer (Cary 50 Bio, Varian) at 535 nm against a solvent reagent blank. The quantity of IAA was expressed in $\mu g\ ml^{-1}$ by referring to a standard graph of IAA, prepared from a series of IAA solutions of known concentrations.

3.11.2. Gibberellic acid (GA_3) production

One ml of the culture at exponential stage was inoculated in LB broth and incubated for 7 days at room temperature. The cultures were centrifuged for 10 min at 10,000 rpm and the supernatant was collected. The cell pellet was re-extracted with phosphate buffer (pH 8.0) and again centrifuged. Both supernatants were collected and pooled, acidified to pH 2.0 with 5 N HCl and extracted with equal volumes of ethyl acetate thrice. The ethyl acetate phase was evaporated at 32°C and the residue was redissolved in 2 ml of distilled water containing 0.05 per cent of Tween 80 (Borrow *et al.*, 1955).

Two ml of zinc acetate solution (21.9 g zinc acetate was dissolved in 80 ml distilled water containing 1 ml glacial acetic acid and made up to 100 ml) was added to the dissolved residue. After 2 min, 2 ml of potassium ferrocyanide solution (10.6 g potassium ferrocyanide dissolved in 100 ml distilled water) was added and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 per cent hydrochloric acid and the mixture was incubated at 20°C for 75 min. The blank was prepared with 5 per cent hydrochloric acid. The absorbance was measured at 254 nm in spectrophotometer (Cary 50 Bio, Varian) (Mahadevan and Sridhar, 1982).

3.11.3. ACC deaminase activity (Honma and Shimomura, 1971)

To measure ACC deaminase activity, diazotrophs were grown in 5 ml of LB medium at 30°C for 2-3 days until they reached stationary phase. To induce ACC deaminase activity, the cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 ml of modified M9 minimal medium supplemented with 5 mM final concentration ACC and incubated at 30°C with shaking for another 36-40 hours. ACC deaminase activity was determined by measuring the production of alpha-ketobutyrate generated by the cleavage of ACC by ACC deaminase. The induced bacterial cells were harvested by centrifugation for 10 min at 10,000 µg, washed twice with 0.1 M Tris-HCl (pH 7.5), and resuspended in 200 µl of 0.1 M Tris-HCl (pH 8.5). The cells were labilized by adding 5% toluene (v/v) and then vortexed at the highest speed for 30 seconds. Fifty µl of labilized cell suspension was incubated with 5 µl of 0.5 M ACC in an microfuge tube at 30°C for 30 min. The negative control for this assay included 50 µl of labilized cell suspension without ACC, while the blank included 50 µl of 0.1 M Tris-HCl (pH 8.5) with 5 µl of 0.5 M ACC. The samples were then mixed thoroughly with 500 µL of 0.56 N HCl by vortexing, and the cell debris was removed by centrifugation at 20,000 X g for 5 minutes.

A 500 µl aliquot of the supernatant was transferred to a 13 X 100 mm glass test tube and mixed with 400 µl of 0.56N HCl and 150 µl of DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 ml of 2N HCl); and the mixture was incubated at 30°C for 30 minutes. One ml of 2N NaOH was added to the sample before the absorbance at 540 nm was measured in spectrophotometer (Cary 50 Bio, Varian). The concentration of α-ketobutyrate in each sample was determined by comparison with a standard curve generated as follows: 500 µl α-ketobutyrate solutions of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM were mixed respectively with 400 µl of 0.56 N HCl and 150 µl DNF solution. One ml of 2N NaOH was added and the absorbances at 540 nm in spectrophotometer (Cary 50 Bio, Varian) were determined as described above. The values for absorbance versus α-ketobutyrate concentration (mM) were used to construct a standard curve. The protein concentration of toluenized cells was determined by the method of Bradford (1976). The ACC deaminase activity was expressed as nmoles of α-ketobutyrate mg of protein per hour.

3.11.4. Siderophore production

Qualitative assay (Schwyn and Neilands, 1987)

Production of siderophores by the bacterial isolates were performed by plate assay. The tertiary complex Chrome azurol S (CAS) / Fe^{3+} / hexadecyl trimethyl ammonium bromide served as an indicator. Seventy two hour old culture of the strains was streaked on to the succinate medium amended with indicator dye. The formation of bright zone with yellowish fluorescent colour by the culture in the dark colored medium indicated siderophore production. The result was scored either positive or negative to this test, based on the colour change of the medium from blue to fluorescent yellow while no colour change marks absence of siderophore production. The isolates with positive to qualitative assay were further analyzed for quantitative assay.

Quantitative assay (Arnow, 1936)

The cultures were grown in modified Fiss minimal medium for seven days. The supernatant was separated and collected by centrifugation for 5 min at 13,500 rpm and was used for Arnow's assay for the detection of catechol-type siderophore. To 1 ml of culture supernatant the following solutions were added in order with constant stirring; 1 ml 0.5 N hydrochloric acid, 1 ml nitrate-molybdate reagent (10 g sodium nitrate and 10 g sodium molybdate in 100 ml distilled water), 1 ml 1 N sodium hydroxide, and distilled water to bring the volume up to 5 ml and incubated at room temperature for 5 min. The absorbance of the solution was measured at 500 nm in a UV VIS spectrophotometer (Cary 50 Bio, Varian).

3.11.5. Hydrogen cyanide (HCN) production

Qualitative assay (Millar and Higgins, 1970)

The bacterial isolates were streaked separately on nutrient agar medium supplemented with 4.4 g glycine l^{-1} and filter paper discs soaked in picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3, 1000 ml of distilled water) was placed in the lid of each Petri dish. Dishes were sealed with Parafilm are incubated at $28^o \pm 1^oC$ for 48 h. A change in colour of the filter paper discs from yellow to light brown, brown or reddish brown was recorded as an

indication of weak, moderate or strong in producing HCN by each strain, respectively. The isolates with positive to qualitative assay were further analyzed for quantitative assay.

Quantitative assay (Lorck, 1948)

The bacterial isolates were grown in 250 ml flasks containing nutrient broth and were incubated at 32 ± 1ºC. Filter paper was cut into uniform strips of 10 cm length and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28 ± 2ºC for 48 h, the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of hydrocyanicacid evolved. The colour was eluted by placing the filter paper strip in a clean test tube containing 10 ml of distilled water and absorbance was measured at 625 nm in a UV VIS spectrophotometer (Cary 50 Bio, Varian). The HCN production was expressed as μg per mg of protein.

3.11.6. Antagonistic activity

The antagonistic activity of all the diazotrophic isolates against 4 plant pathogenic organism *viz.*, *Xanthomonas oryzae* (bacterial leaf blight), *Rhizoctonia solani* (sheath blight),*Pyricularia oryzae* (blast) and *Sarocladium oryzae* (sheath rot) were evaluated. A mycelial disc of the fungal pathogen (5 mm) was placed at one end of the Petri plate containg PDA medium. The bacterial antagonists were streaked 1 cm away from the periphery of the Petri plate just opposite to the mycelial disc of the pathogen by dual culture technique (Dennis and Webster, 1971). For bacterial leaf blight (*X. oryzae*) disc diffusion assay (Perez *et al*., 1990) was used to check the antagonistic activity of all the diazotrophic isolates. Visual observation on the inhibition of the growth of pathogenic organism was recorded after 96 h of incubation in comparison with the control PDA plate simultaneously inoculated with fungal pathogen only. The inhibition of the growth of pathogenic organism was recorded as positive.

3.12.6. Mineral solubilization by diazotrophic isolates from selected grass species

3.12.6.1. Assessment of phosphorus solubilization

Qualitative assay

The bacterial cultures were inoculated in to hydroxy apetite medium (Sperber, 1958). The test organisms were inoculated on these media and incubated (Lab Companion, Korea) at 30°C for 48 h. The diameter of the clearing zones around the

colonies were measured. The solubilizing efficiency was calculated as indicated below (Srivastav *et al*., 2004).

$$\text{Solubilization efficiency (\%)} = \frac{\text{Diameter of solubilization zone – colony diameter}}{\text{Colony diameter}} \times 100$$

Quantitative assay

One ml of the culture containing 10^9 cell ml^{-1} was inoculated into the flasks of Pikovaskaya's broth containing 100 mg of tricalcium phosphate. An uninoculated control was maintained. After 7 days incubation, the contents were centrifuged at 7000 rpm for 10 min and clear supernatant was used for soluble P estimation following method described by Olsen *et al*. (1954). One ml of the culture filtrate was pipette into a 25 ml volumetric flask and diluted to 20 ml with water. Four ml of reagent (1.056 g of ascorbic acid in 200 ml of reagent A) was added and the volume was made up to 25 ml with distilled water. The intensity of blue colour was read in spectrophotometer (Cary 50 Bio, Varian) at 660 nm. The standard curve was prepared with orthophosphate (KH_2PO_4) and amount of P solubilized was calculated by referring to standard graph. The phosphorus content was expressed in terms of mg of phosphorus ml^{-1}. Influence of phosphorus solubilizing organisms on the pH and titrable acidity of growth medium was also analyzed as per the standard protocol. The available P was expressed as mg ml^{-1}.

Phosphatase assay

The phosphatase activity was determined based on the liberation of p-nitrophenol from p-nitrophenol phosphate by colorimetric method (Morton, 1952). The positive isolates of above assay were grown in Pikovskaya's medium for 24 h in an environmental shaker. The phosphatase activity was estimated by adding 1 ml of substrate solution (100 mg of p-nitrophenol phosphate in 100 ml distilled water), 10 ml of acetate buffer (Annexure II) and 2 ml of enzyme source. The contents were thoroughly mixed and incubated at room temperature. After 24 h of incubation, 10 ml of assay mixture was withdrawn, centrifuged at 5000 rpm for 15 min. 1 ml of supernatant was mixed with 1 ml of fresh Folin's reagent (prepared by mixing one part of Folin-ciocalteau reagent and one part of distilled water) and 2 ml of 20 per cent sodium carbonate were added and boiled exactly for 1 min. It was

immediately removed and the volume was made up to 10 ml and the colour was read in spectrophotometer (Cary 50 Bio, Varian) at 600 nm. The phosphatase activity was calculated and expressed as µg of p-nitrophenol released per ml of culture filtrate.

3.12.6.2. Assessment of potassium releasing potential

Qualitative assay

Bacterial cultures were streaked in the modified Aleksandrov medium containing 0.25 per cent potassium aluminium silicate as insoluble source for potassium solubilization. The plates were incubated in incubator (Lab Companion, Korea) at room temperature for 48 h. The colonies exhibiting clear zones around them were considered as positive result of solubilization.

Quantitative assay (Saxena, 1989)

The positive isolates of above assay were further assessed by growing the culture in basal liquid medium supplemented with 0.25 per cent potassium aluminium silicate. The culture containing 2×10^9 cells ml^{-1} was inoculated into 50 ml broth taken in Erlenmeyer flask and incubated at room temperature. An uninoculated control was maintained. After 7 days incubation, the contents were filtered and centrifuged to remove cells and debris. The clear supernatant was analysed for the potassium content using flame photometric method (Systronics flame photometer 128). 5 ml of culture filtrate was taken in polythene beakers to which 1 ml of reagent A (50 ml of conc. HCl was mixed with 50 ml of distilled water) and 2 ml of reagent B (Ammonium molybdate, 10 g was dissolved in 100 ml of distilled water) were added. After 10 min, 1.5 ml of reagent C (Oxalic acid, 10 g was dissolved in 100 ml of distilled water) was added and mixed thoroughly. The yellow colour developed was read at 420 nm in flame photometer. The unknown were calculated from a standard curve. Influence of potassium solubilizing organisms on the pH and titrable acidity of growth medium was also analyzed as per the standard protocol. The available K was expressed as mg ml^{-1}.

3.12.6.3. Assessment of zinc solubilization

The zinc solubilization potential of the isolates was assessed both qualitatively and quantitatively under *in vitro* conditions.

Qualitative assay

The bacterial cultures were inoculated into Bunt and Rovira agar medium containing 0.1 per cent of ZnO (Bunt and Rovira, 1955). The test organisms were inoculated on these media and incubated (Lab Companion, Korea) at 30°C for 48 h. The diameter of the clearing zones around the colonies was measured. The solubilizing efficiency was calculated (Srivastav *et al*., 2004).

Quantitative assay

The positive isolates of above assay were further analyzed for their Zn solubilizing efficiency by growing them in 100 ml Erlenmeyer flasks containing 50 ml of basal liquid medium supplemented with 0.1percent ZnO. In this, 1 ml of 36 h old broth culture containing 1×10^9 cells ml^{-1} of various isolates was inoculated. Appropriate uninoculated controls were maintained and all the treatments were replicated. The bacterial cultures were withdrawn after 7^{th} day of incubation at room temperature for the estimation of soluble Zn. The bacterial cultures were centrifuged at 10,000 rpm for 10 min to remove the debris and cells. Ten ml of this solution was fed to Atomic Absorption Spectrometer (Varian H 240, Australia) to determine the available zinc content (Manib *et al.*, 1986). Influence of zinc solubilizing organisms on the pH and titrable acidity of growth medium was also analyzed as per the standard protocol. The available Zn was expressed as mg ml^{-1}.

3.13. Stress tolerance by diazotrophic isolates from selected grass species

3.13.1. Salt tolerance

The diazotrophic cultures were inoculated on LB broth containing different concentrations of NaCl *viz.*, 1, 2.5, 5 and 7.5 per cent and incubated in incubator (Lab Companion, Korea) at 37ºC for 2 days. The population was analyzed by serial dilution and plating at 12 h interval upto 48 h and expressed as the number of viable cells ml^{-1}.

3.13.2. Temperature tolerance

To analyse the temperature tolerance of the diazotrophic isolates, the cultures were inoculated on LB broth and incubated in incubator (Lab Companion, Korea) at different temperatures *viz.*, 40ºC, 45ºC, 50ºC and 55ºC for 2 days. The population was analyzed by serial dilution and plating at 12 h interval upto 48 h and expressed as number of viable cells ml^{-1}.

3.13.3. Dessication tolerance

Oven dried soil (10g) was placed in 30 ml screw capped vial. Soil was sterilized by autoclaving (Tomy, Japan) at 121^{o}C for 2h on two successive days. Soil in these vials were maintained at two moisture levels *viz.*, field capacity (1BAR) and permanent wilting point (15 BAR) based on soil moisture release curve determined by the pressure plate method (Richards, 1954) using pressure plate apparatus.

Each vial was adjusted to one of the two moisture levels by inoculating (10^9 cells ml^{-1}) of inoculum and a variable volume of sterile water. The inoculated soil was thoroughly mixed and incubated at 37^{o}C in an incubator (Lab Companion, Korea). Each treatment was replicated three times with a completely randomized design. The number of viable cells was determined at 15 days intervals by serial dilution and plating on LB agar medium up to 60 days. Population were expressed as number of viable cells g^{-1} soil.

3.13.4. Heavy metal tolerance

The maximum tolerance concentration (MTC) of various heavy metals by diazotrophic isolates was determined. For this, stock solutions of selected heavy metals *viz.*, Cd^{2+} , Hg^{2+} , Zn^{2+} ,Co^{2+} and Ni^{2+} were prepared by dissolving the respective salts i.e. $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, $ZnSo_4$, $CoCl_2$ and $NiCl_2$ in distilled water so as to obtain µg ml^{-1} concentration of each heavy metal. From this, appropriate quantity was added to LB medium to obtain 100, 200, 400, 600, 800 and 1000 µg ml^{-1} concentration. The cultures were streaked on to LB plates and incubated at 30°C for 4 days and growth was observed.

3.14. Impact of diazotrophic isolates with multifaceted growth promoting activity on the growth of rice plants under *in vitro* condition

To study the impact of diazotrophic isolates on the growth of rice plants, 15 isolates were selected based on the multiple beneficial characters *viz*., nitrogenase activity, presence of *nifH* gene, production of plant growth promoting substances, antagonistic activity and tolerance level to various stress conditions.

Dehulled seeds of rice (cultivar- ADT 43) were surface sterilized by immersion in 70 % ethanol for 30 seconds, followed by soaking in 0.2 % mercuric chloride for 30 seconds and then washed with several times with sterilized distilled water. The surface sterilized

seeds were germinated aseptically in 1 % sucrose agar medium. Three days old seedlings that were free of any visual bacterial and fungal contamination were used for inoculation with diazotrophic isolates. The elite multi-functional diazotrophic trains were grown in LB broth (Annexure II) till the population reached to 10^{10} cells ml^{-1}. The cells were then harvested by centrifugation at 6000 rpm for 5 min at room temperature. The cell pellets were washed twice with 20 ml of phosphate buffer and resuspended in 1.5 ml of phosphate buffer. Seeds were treated with the selected bacterial inoculants for 15 min. Pre-germinated seeds were placed at the rate of one seed in each 200 ml boiling tube containing 40 ml of N-free Fahraeus medium. The seedlings were grown in a growth chamber at 27ºC (HECO plant growth chamber). An uninoculated plant served as control.

3.14.1. Biometric observation of rice seedlings

The whole plant from each treatment was uprooted on 21 DAS after sowing, dried in sunlight for 2 days and then dried in an oven at 60ºC until constant weight was obtained and weight was expressed in mg $plant^{-1}$. The shoot length of the rice plant was measured from the collar region to the tip of the plant and expressed in cm and the root length was measured from the collar region to the bottom of the root and expressed in cm.

3.14.2. Observation of endophytic bacterial colonization of rice seedlings by scanning electron microscopy (SEM)

The roots and culms from 15 days old fresh rice seedlings were cut, fixed with 3 % (v/v) glutaraldehyde, for 2 h at 4°C and washed with 0.1M phosphate buffer (pH 7.2) at room temperature for 10 min (three times). The samples again post fixed in 1% (w/v) osmium tetroxide in the same buffer for 2 h at 4°C. The fixed samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 75% and 100%) for 5 minutes in each concentration. Then the samples were treated with CO_2 and mounted on an aluminum cylinder with silver paste, and finally covered with a steam of carbon and ionized gold (Nowell and Parules, 1980). The samples were examined under a SEM (ICON, anaytical FEI Quanta 200, USA) operated at 15 kV at an 8-10 mm distance. Colonizing ability of the endophytic isolates in rice seedlings were documented as microphotographs.

3.15. Effect of elite diazotrophic isolates on the growth of rice (variety- ADT 43) under field condition

Field experiment was conducted in the Wetland Farm, Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore during *rabi* (February-may) 2013. The experiment was carried out in randomized block design (RBD) with three replications for each treatment. The treatment details are given below.

T1-*Klebsiella* sp.(CG1)+75% RDF	T7- *Serratia* sp. (CRE9)+75% RDF
T2-*Bacillus* sp. (CG5)+75% RDF	T8- *Pseudomonas* sp.(CRE10)+75% RDF
T3- *S. marcescens* (CD1)+75% RDF	T9- *P. agglomerans* (ORE9)+75% RDF
T4- *Serratia* sp. (CB2)+75% RDF	T10- *A. lipoferum* (Az 204)*+75%RDF
T5- *K. pneumoniae* (CR2)+75% RDF	T11-100% RDF
T6- *Serratia* sp.(OR3)+75% RDF	T12-Control (Absolute control)

Physico-chemical properties of experimental field were, pH-8.17, electrical conductivity- 4.32 dSm^{-1}, organic carbon- 0.99%, available nitrogen-225.6 $kgha^{-1}$, available phosphorus- 18.6 kg ha^{-1} and available potassium-345.7 kg ha^{-1}. The experimental field was prepared as per the recommendations. Fourteen days old seedlings obtained from nursery were transplanted at the rate of one seedling $hill^{-1}$ at 25 cm x 25 cm spacing. Irrigation, weed management and plant protection were done as per the recommendation whenever applicable. The elite multi-functional diazotrophic strains were grown in LB broth (Annexure II) till the population reached to 10^{10} cells ml^{-1}. Seeds were treated with the above bacterial inoculants after preparing semisolid slurry by mixing with carboxy methyl cellulose as an adhesive and shade dried for 30 min before sowing. The crop was harvested when most of the plants turned yellow and attained physiological maturity. The biometric observations were recorded at tillering, panicle initiation, flowering and harvesting stages of the crop. For assessing the effect of diazotrophic isolates on yield attributes such as panicle length (cm), test weight (g), grain yield (kg ha^{-1}) and straw yield (kg ha^{-1}) were recorded at the time of harvest. Soil enzyme activity *viz*., fluorescein diacetate (FDA) hydrolysis, arginine deaminase (ADA) activity

were measured as described in 3.5. The nitrogen content in the plant sample on dry weight basis was estimated by Micro Kjeldhal Method as suggested by Subbiah and Asija (1956) and expressed as kg ha^{-1}.

3.16. Statistical analyses

All the data were subjected to statistical analysis with softwares, SPSS (Kirkpatrick and Feenay, 2005) and Microsoft Excel for Windows 2007 add-ins with XLSTAT Version 2010.5.05 (XLSTAT, 2010). Statistically significant differences between the treatments were analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at 5 % significance level. To reveal similarities and differences between sample plots and the relationships between different variables, principal component analysis (PCA) (Wold *et al*., 1987) was performed on all data. In a PCA model, the objects (samples) are represented by their scores and the variables are represented by their loadings. The scores and loadings can be presented in a graph where two components are plotted against each other. In a score plot, similar samples will be positioned close to each other and in loading plots, positively correlated variables will be positioned close to each other and negatively correlated will be opposite to each other. Samples that are high in a specific variable will be pulled towards the area of the score plot where the variable in the corresponding loading plot is located. The number of variables was reduced by excluding those explained to less than 50 % by the significant components.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

In the present study, diazotrophic bacteria were isolated from rhizosphere and internal tissuses of ten selected grass species. The diazotrophic bacteria were screened and identified using 16S rRNA gene sequencing. The presence of *nif* gene was detected by PCR using universal primers. These cultures were found to produce multiple plant growth promoting substances and tolerance to various abiotic stresses. Gnotobiotic experiment was conducted to screen the efficient diazotrophic isolates on the growth of rice. Further, the efficient cultures were evaluated for their performance in rice by field experiment. The results obtained are presented below.

4.1. Physio-chemical and biological properties of rhizosphere and non-rhizosphere soil samples obtained from selected grass species

Based on their predominance in each region, a total of 10 different grass species *viz ., Brachiaria reptans, Cenchrus glaucus, Saccharum spontaneum, Panicum repens, Cyperus rotundus, Dactyloctenium aegyptium, Chloris barbata, Oryza rufipogon, Cyanodon dactylon* and *Setaria verticillata* along with the soil (rhizosphere and non - rhizosphere) were sampled (Fig. 3 and Plate1).

The samples collected from different locations were analysed for soil reaction and salt concentrations. Accordingly soils were grouped as slightly acidic, highly acidic, alkaline, saline, saline –sodic and acid sulphate. The various grass species that dominated the different soil groups are *C. glaucus* (slightly acidic), *O. rufipogon* (highly acidic), *C.dactylon* (alkaline), *B. reptans, S. spontaneum, P. repens, C. Barbata, S. verticillata* (saline), *C. rotundus* (saline-sodic) and *D. aegyptium* (acid sulphate) (Table 4).

The soil microbial population of selected grass species from different regions were enumerated and presented in Table 5. From the results, it was found that the rhizosphere soil samples from *B. reptans* harboured more number of bacteria (8.64 ± 0.18 log cfu g^{-1} dry weight of soil) and actinobacteria (4.30 ± 0.81 log cfu g^{-1} dry weight of soil). The maximum fungal population (5.31 ± 0.31 log cfu g^{-1} dry weight of soil) and diazotrophs (5.18 ± 0.53 log cfu g^{-1} dry weight of soil) were recorded from *P. repens* and

Table 4. Physio-chemical properties of rhizosphere and bulk soil samples of grass species collected from different physiographic regions

Grass species	Source	pH	EC (dSm^{-1})	ESP %	Available Nutrients (kg ha^{-1})		
					N	P	K
B. reptans	R	7.2 (± 0.18)^{i}	4.5 (± 0.23)^{e}	10.5 (± 0.67)^{e}	161.4 (± 23.5)^{c}	27.5 (± 12.6)^{g}	134.2 (± 13.8)^{p}
	B	7.4 (± 0.28)^{gh}	4.6 (± 0.24) ^{de}	10.7 (± 0.34)^{d}	150.4 (± 21.5)^{f}	23.5 (± 12.8)^{m}	127.2 (± 12.2)^{r}
C. glaucus	R	6.2 (± 0.08)^{j}	2.7 (± 0.98)^{g}	4.2 (± 0.34)^{l}	128.8 (± 13.1)^{p}	40.5 (± 12.7)^{a}	295.3 (± 24.1)^{a}
	B	6.2 (± 0.08)^{j}	2.7 (± 0.98)^{g}	4.2 (± 0.34)^{l}	126.5 (± 12.5)^{q}	39.5 (± 13.7)^{b}	285.3 (± 23.1)^{b}
S. spontaneum	R	8.2 (± 0.28)^{d}	4.5 (± 0.56)^{e}	8.1 (± 0.45)^{i}	184.7 (± 12.8)^{a}	39.3 (± 14.7)^{c}	230.5 (± 45.7)^{e}
	B	8.5 (± 0.26)^{c}	4.6 (± 0.36)^{de}	8.3 (± 0.45)^{gh}	180.7 (± 23.8)^{b}	34.2 (± 12.7)^{d}	213.5 (± 47.9)^{g}
Panicum repens	R	7.3 ± 0.23)^{hi}	4.7 (± 0.14)^{d}	8.5 (± 0.42)^{f}	119.8 (± 15.9)^{r}	19.5 (± 16.9)^{p}	117.4 (± 13.5)^{s}
	B	7.5 ± 0.45)^{fg}	4.5 (± 0.13)^{e}	8.5 (± 0.23)^{f}	103.8 (± 12.9)^{s}	14.5 (± 12.9)^{s}	109.4 (± 11.4)^{t}
C. rotundus	R	8.7 (± 0.22)^{ab}	4.2 (± 0.12)^{f}	18.5 (± 0.41)^{c}	156.8 (± 23.6)^{e}	29.0 (± 18.9)^{e}	159.5 (± 15.2)^{m}
	B	8.8 (± 0.32)^{a}	4.3 (± 0.24)^{f}	18.5 (± 0.54)^{c}	128.8 (± 16.8)^{p}	27.0 (± 17.9)^{h}	134.5 (± 12.1)^{q}
D.aegyptium	R	3.8 (± 0.26)^{m}	0.8 (± 0.06)^{h}	1.2 (± 0.78)^{o}	140.5(± 16.8)^{k}	25.0 (± 15.9)^{j}	188.7 (± 14.4)^{i}
	B	3.9 (± 0.26)^{m}	0.8 (± 0.07)^{h}	1.3 (± 0.78)^{o}	138.6 (± 1.11)^{l}	23.4 (± 16.7)^{m}	180.7 (± 12.4)^{j}
C.barbata	R	7.7 (± 0.45)^{e}	6.3 (± 0.24)^{b}	7.5 (± 0.45)^{k}	149.5 (± 23.6)^{h}	28.0 (± 16.8)^{f}	214.9 (± 22.7)^{f}
	B	7.7 (± 0.45)^{e}	6.6 (± 0.24)^{a}	7.7 (± 0.45)^{j}	147.5 (± 46.6)^{j}	22.6 (± 16.8)^{n}	213.9 (± 21.5)^{g}
O.rufipogon	R	5.3 (± 0.67)^{l}	0.72 (± 0.04)^{h}	1.5 (± 0.35)^{n}	132.4 (± 12.7)^{n}	26.8 (± 14.4)^{i}	170.4 (± 23.8)^{k}
	B	5.6 (± 0.57)^{k}	0.77 (± 0.04)^{h}	1.8 (± 0.38)^{m}	130.4 (± 10.7)^{o}	24.8 (± 12.6)^{l}	168.3 (± 21.9)^{l}
C.dactylon	R	8.6 (± 0.18)^{bc}	0.18 (± 0.06)^{i}	25.0 (± 0.13)^{b}	152.4 (± 16.8)^{f}	19.3 (± 12.6)^{q}	278.5 (± 13.1)^{c}
	B	8.8 (± 0.18)^{a}	0.20 (± 0.08)^{i}	27.0 (± 0.34)^{a}	160.4 (± 16.8)^{d}	18.3 (± 10.4)^{r}	268.9 (± 12.1)^{d}
S.verticillata	R	7.5 (± 0.89)^{fg}	5.8 (± 0.67)^{c}	8.2 (± 0.67)^{hi}	148.3 (± 18.9)^{i}	25.5 (± 32.6)^{i}	154.6 (± 31.8)^{n}
	B	7.6 (± 0.95)^{ef}	5.7 (± 0.78)^{c}	8.4 (± 0.79)^{fg}	133.3 (± 18.9)^{m}	21.5 (± 32.6)^{o}	143.5 (± 32.8)^{o}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); R- Rhizosphere; B-Bulk soil; EC- Electrical conductivity ; ESP- Exchangeable sodium percentage

Table 5. Population of culturable soil microbial communities from rhizosphere and bulk soil samples of grass species collected from different physiographic regions

Grass species	Source	Culturable soil microbial population (log cfu g^{-1} dry weight of soil)			
		Bacteria	Fungi	Actinobacteria	Diazotrophs
B. reptans	R	8.64 (± 0.18)^{a}	5.23 (± 0.23)^{ab}	4.30 (± 0.81)^{a}	5.01 (± 0.45)^{ab}
	B	8.29 (± 0.34)$^{a-f}$	5.12 (± 0.20)$^{b-g}$	4.05 (± 0.20)^{b}	4.48 (± 0.51)^{h}
C. glaucus	R	8.38 (± 0.08)^{abc}	5.13 (± 0.10)$^{b-g}$	3.60 (± 0.15)^{f}	5.03 (± 0.40)^{ab}
	B	8.23 (± 0.38)$^{c-g}$	5.08 (± 0.12)$^{b-g}$	3.30 (± 0.08)^{g}	4.60 (± 0.45)^{fg}
S. spontaneum	R	8.35 (±0.25)$^{a-d}$	5.11 (± 0.13)$^{b-g}$	3.91 (± 0.73)^{bcd}	4.97 (± 0.45)$^{a-c}$
	B	8.16 (± 0.88)^{efg}	4.98 (± 0.20)^{fg}	3.90 (± 0.21)^{bcd}	4.70 (± 0.58)^{fg}
P. repens	R	8.44 (± 0.03)^{ab}	5.31 (± 0.31)^{a}	3.92 (± 0.58)^{bc}	4.72 (± 3.53)^{efg}
	B	8.12 (± 0.86)^{fg}	5.04 (± 0.36)$^{d-g}$	3.72 (± 0.45)^{ef}	4.78 (± 0.15)^{def}
C. rotundus	R	8.08 (± 0.58)^{gh}	5.16 (± 3.18)$^{a-e}$	3.70 (± 0.52)^{ef}	4.82 (± 0.28)$^{c-f}$
	B	7.95 (± 0.15)^{h}	5.03 (±0.23)$^{d-g}$	3.85 (± 0.53)^{cde}	4.75 (± 0.84)^{def}
D. aegyptium	R	8.16 (±1.25)^{efg}	5.20 (± 0.33)^{abc}	3.92 (± 2.33)^{bcd}	5.03 (± 0.28)^{ab}
	B	7.93 (± 0.28)^{h}	5.03 (± 0.56)^{fgh}	3.80 (± 0.87)^{cde}	4.90 (± 0.55)$^{b-d}$
C. barbata	R	8.40 (± 0.20)^{ab}	5.18 (± 0.24)$^{a-d}$	3.95 (± 0.65)^{abc}	5.03 (± 0.24)^{ab}
	B	8.24 (± 0.82)^{efg}	4.96 (± 0.20)^{fg}	3.79 (± 0.67)^{cde}	4.78(± 0.56)^{def}
O. rufipogon	R	8.20 (± 0.73)$^{d-g}$	5.08 (± 0.15)$^{b-g}$	3.98 (± 0.73)^{b}	5.18 (± 0.53)^{a}
	B	8.09 (± 0.45)^{gh}	4.97 (± 0.18)^{g}	3.93 (±0 .58)^{bc}	4.75 (± 0.76)^{def}
C. dactylon	R	8.27 (± 0.23)$^{a-f}$	5.04 (± 0.35)$^{c-g}$	3.78 (± 0.08)^{ef}	4.98 (± 0.20)^{abc}
	B	8.16 (± 0.25)^{efg}	5.01 (±0.23)^{efg}	3.70 (± 0.53)^{fg}	4.80 (± 0.86)$^{c-f}$
S. verticillata	R	8.37 (±0.45)^{abc}	5.15 (± 0.35)$^{a-e}$	3.82 (± 0.32)^{cde}	4.97 (± 0.42)^{abc}
	B	8.31 (± 0.20)$^{a-e}$	5.02 (±0.16)^{efg}	3.78 (± 0.43)^{de}	4.88 (± 0.25)$^{b-e}$

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); R- Rhizosphere; B-Bulk soil.

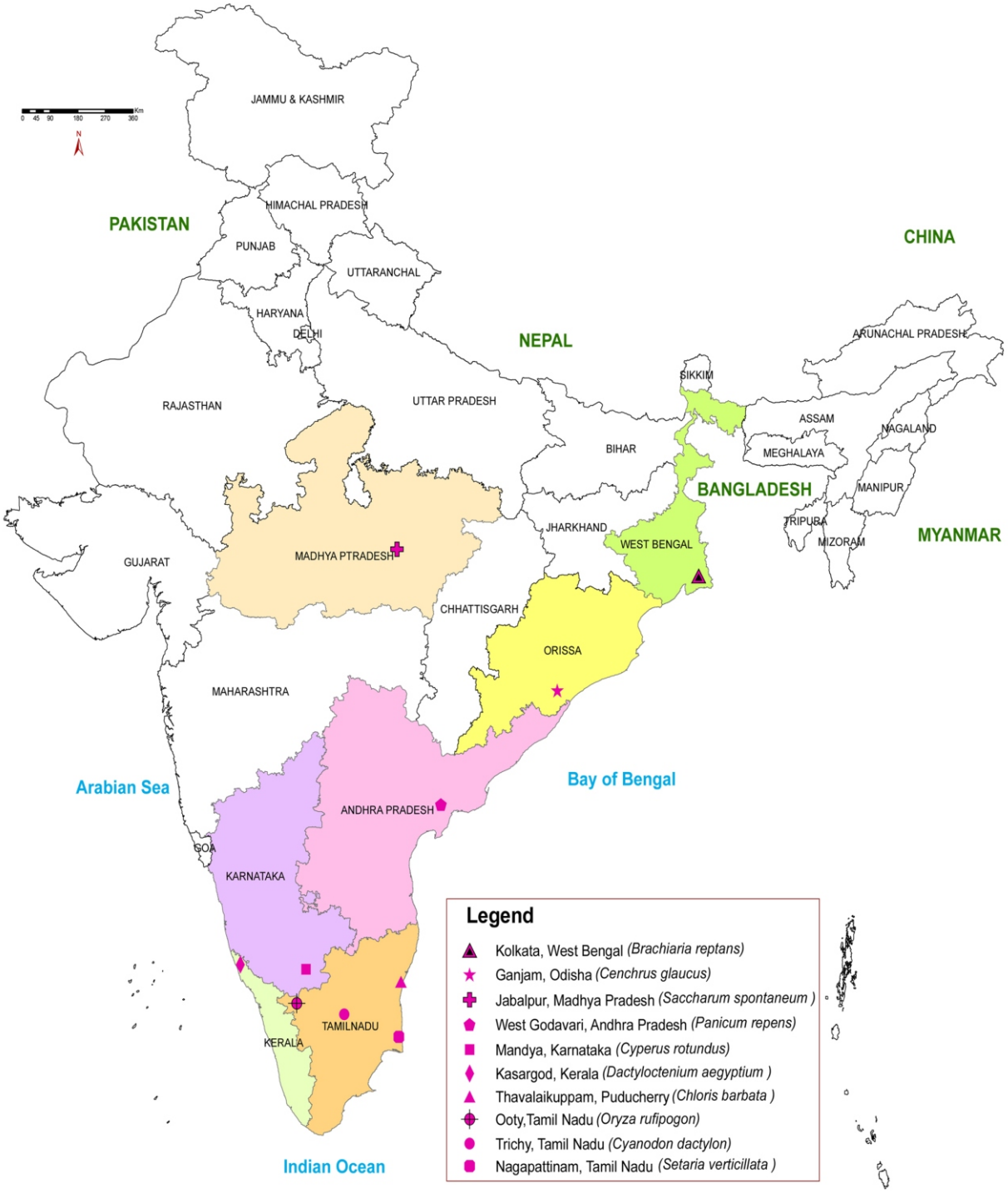

Fig.3. Geographical map showing the sampling sites used in the present study. The grass species collected from the location was given in parenthesis

A

B

C

D

E

F

G

H

I

J

Plate 1. Grass species selected for analysing the diazotrophic diversity. A. *Brachiaria reptans* B. *Cenchrus glaucus;* C. *Saccharum spontaneum;* D. *Panicum repens ;* E. *Cyperus rotundus;* F. *Cyanodon dactylon;* G. *Oryza rufipogon;* H. *Chloris barbata;* I. *Setaria verticillata;* J. *Dactyloctenium aegyptium*

O. rufipogon respectively. In general the rhizosphere soil of grass species harboured more number of total bacteria, fungi, actinobacteria and diazotrophs.

The soil biochemical activity was quantified by using fluorescein diacetate (FDA) hydrolysis and arginine deaminase (ADA) activity. The FDA hydrolysis rate was higher in the rhizosphere soil of *C. glaucus* (14.09 ±0.30 µg fluorescein $g^{-1}h^{-1}$) and *C. rotundus* (13.88 ±0.16 µg fluorescein g^{-1} h^{-1}) .While, the least in the bulk soil of *S. verticillata* (2.22 ±0.02 µg fluorescein g^{-1} h^{-1}) (Fig. 4A and B).

The arginine deaminase activity was found to be maximum in rhizosphere soil of *C. glaucus* (4.4 ±0.17 µg ammonia released g^{-1} soil h^{-1}) which was on par with rhizosphere soil of *O. rufipogon* (4.3 ±0.09 µg ammonia released g^{-1} soil h^{-1}). The lower arginine deaminase activity were observed in bulk soil of *S. verticillata* (2.0 ±0.32 µg ammonia released g^{-1} soil h^{-1}). The soil biochemical activity were found to be less with the bulk soil when compared to rhizosphere soil of all the samples tested (Fig. 4A and B).

4.2. Isolation of rhizosphere and putative endophytic diazotrophs from selected grass species

Sixty diazotrophic isolates were obtained by using four N-free media after 5 days of incubation (Table 6). The highest number of diazotrophs were isolated from the soil samples of *O. rufipogon* and NFMM (N-free malate medium) gave the maximum amount of diazotrophic isolates (32) followed by LGI (28) medium.

Thirty-six of endophytic isolates were obtained from the tissues of ten selected grass species and Rennie semisolid media gave the maximum amount of endophytes (12). These 36 isolates were used for further re-infection study (Koch's postulates). Only 22 bacterial isolates were re-isolated from gnotobiotically grown rice seedlings (Table 7).

Among the twenty two isolates, seven isolates were positive for pectinase activity, ten were able to grow on cellulose medium and 12 isolates showed the motility in semisolid agar medium. Six isolates recorded positive for both cellulase and pectinase activity and only one isolate (ORE7) was positive for all the three activity (Table 8).

Table 6. Selected diazotrophic isolates from rhizosphere of grass species collected from different physiographic regions

Grass species	Isolate code	Isolation media	Number of diazotrophs	Total number of diazotrophs
B. reptans	BR	NFMM	3	5
		LGI	1	
		JNFb	1	
C. glaucus	CG	NFMM	5	7
		LGI	2	
S. spontaneum	SS	NFMM	3	5
		LGI	1	
		TDM	1	
P .repens	PR	NFMM	2	5
		LGI	2	
		JNFb	1	
C. rotundus	CR	NFMM	3	6
		LGI	3	
D. aegyptium	DA	JNFb	1	2
		TDM	1	
C. barbata	CB	NFMM	3	7
		LGI	2	
		JNFb	2	
O. rufipogon	OR	NFMM	5	10
		LGI	3	
		JNFb	2	
C. dactylon	CD	NFMM	4	7
		LGI	3	
S. verticillata	SV	NFMM	4	6
		TDM	2	

NFMM - N free malic acid medium; Nfb- N free bromothymol blue medium;
JNFb - Junior N free bromothymol blue medium; DM-total diazotroph medium

Table 7. Selection of putative endophytic diazotrophs from grass species collected from different physiographic regions

Sample name	Isolate code	Isolation media	Diazotrophic endophytes	Putative diazotrophic endophytes	Total number of putative diazotrophs
B. reptans	BRE	Nfb	1	0	0
		Rennie	1	0	
C. glaucus	CGE	Nfb	3	2	4
		Rennie	2	2	
S. spontaneum	SSE	JNfb	2	1	1
P. repens	PRE	Nfb	2	1	2
		LGI	2	1	
C. rotundus	CRE	Nfb	3	2	4
		Rennie	2	2	
D. aegyptium	DAE	LGI	1	0	0
C. barbata	CBE	LGI	2	1	2
		Rennie	1	1	
O. rufipogon	ORE	SM	4	2	7
		SSM	1	1	
		Rennie	4	4	
C. dactylon	CDE	LGI	2	1	1
		Rennie	2	0	
S. verticillata	SVE	JNfb	1	1	1

Nfb - N free bromothymol blue medium; JNFb- Junior N free bromothymol blue medium; SM- Semi solid malate medium; SSM -Soil saturation extract semi solid malate medium

Table 8. Characterization of the putative endophytic diazotrophs from grass species collected from different physiographic regions

Isolates	Cellulase	Pectinase	Motility
CGE1	+	+	-
CGE2	+	-	+
CGE3	+	+	-
CGE4	-	-	+
SSE5	-	-	-
PRE1	-	-	+
PRE2	-	-	-
CRE7	-	-	+
CRE8	+	+	-
CRE9	-	-	-
CRE10	-	-	+
CBE8	+	-	-
CBE9	+	+	-
CBE10	-	+	+
ORE7	+	+	+
ORE9	+	-	+
ORE10	-	-	-
ORE11	+	-	-
ORE12	+	-	-
ORE13	+	+	-
CDE4	-	-	+
SVE9	-	-	+
G.diazotrophicus (PAL5)*****	+	+	+

+ positive; - Negative; *standard strain

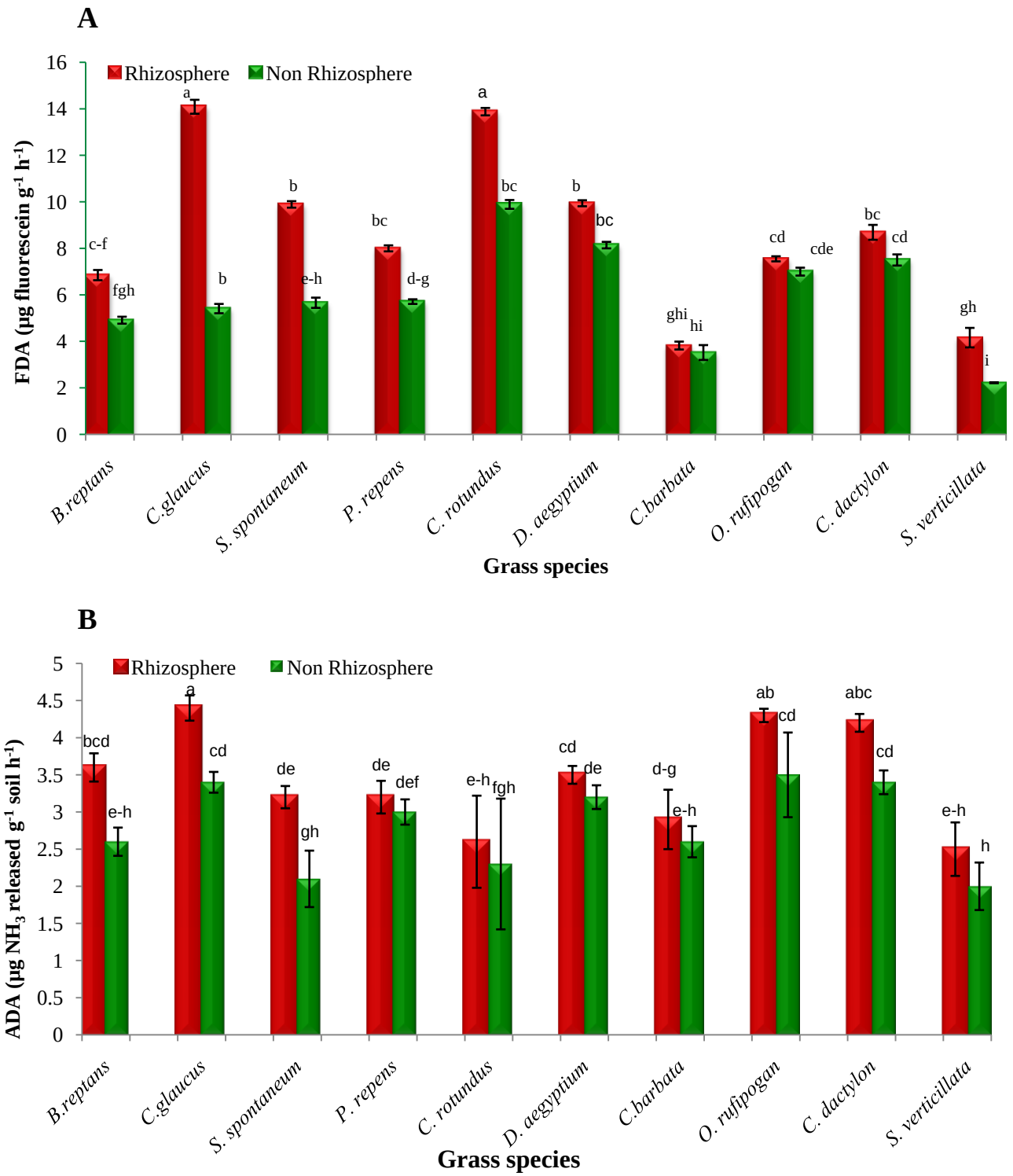

Fig. 4. Flourescein diacetate (A) and Arginine deaminase (B) activity of rhizosphere and bulk soils of grass species. Data are presented as mean ± SE(n=3); For each panel, different letters indicate significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

4.3. Preliminary screening for diazotrophy of the isolates obtained from selected grass species

4.3.1. Growth of diazotrophic isolates in N- free medium

The diazotrophic isolates from ten selected grass species were tested for their ability to grow in N-free medium as qualitative evidence of the atmospheric nitrogen fixation (Table 9). Out of sixty diazotrophic isolates, thirty seven isolates recorded positive growth in N- free medium whereas among the twenty two endophytic isolates, twenty one isolates recorded positive growth in N-free medium (Table 9).

The isolates with positive growth in N- free medium were further analyzed for the presence of total Nitrogen and ammonia.

4.3.2. Total Nitrogen (N) and ammonia production by the diazotrophic isolates

Total N of rhizosphere diazotrophs varied from 1.8±0.36 to 14.7±0.12 mg N $g^{-1}$of malate. Among the rhizosphere diazotrophs tested, isolate CD1 produce the maximum amount (14.7 ± 0.12 mg N g^{-1} of malate) of nitrogen. The isolate BR2 (1.8 ± 0.36 mg N g^{-1} of malate) produced the least amount of total nitrogen. The quantity of ammonia production by rhizosphere diazotrophs varied between 1.1± 0.04 to 4.2 ± 0.26 mg ml^{-1}. Among the isolates, CB2 produced maximum quantity of ammonia (4.2 ± 0.24 mg ml^{-1}) (Table 10).

In endophytic isolates, CRE9 was found to produce the highest amount (11.7±0.23 mg N g^{-1} of malate) of total N content. The quantity of ammonia production varied between 1.1 ± 0.08 to 5.4 ± 0.37 mg ml^{-1}. The endophytic isolate CRE10 (5.4±0.37mg ml^{-1}) registered the maximum amount of ammonia production (Table 11).

Based on total nitrogen and ammonia production, twenty eight rhizosphere isolates and twenty endophytic diazotrophs were selected for secondary screening.

4.4. Secondary screening for diazotrophy of the isolates obtained from selected grass species

4.4.1. Nitrogenase enzyme activity of diazotrophic isolates

The nitrogen fixing ability was determined by acetylene reduction assay and the results are reported in Table 12 and 13. The nitrogenase enzyme activity of the rhizosphere

Table 9. Growth pattern of rhizosphere diazotrophic isolates in N- free medium

Isolate	Growth (A_{660nm})	Isolate	Growth (A_{660nm})	Isolate	Growth (A_{660nm})
Rhizosphere diazotrophs					
BR1	+++	CR2	++	OR5	+
BR2	+	CR3	++	OR6	+
BR3	-	CR4	-	OR7	+
BR4	-	CR5	-	OR8	+
BR5	-	CR6	-	OR9	+
CG1	++	DA1	-	CD1	++
CG2	-	DA2	++	CD2	+++
CG3	+++	DA3	-	CD3	++
CG4	+	DA4	-	CD4	-
CG5	+	DA5	+	CD5	-
SS1	-	CB1	++	CD6	-
SS2	-	CB2	+++	CD7	-
SS3	-	CB3	++	SV1	++
SS4	++	CB4	++	SV2	+
SS5	-	CB5	+++	SV3	+
PR1	++	CB6	+	SV4	+
PR2	++	CB7	+	SV5	-
PR3	-	OR1	++	SV6	-
PR4	-	OR2	+++	*A.lipoferum* (Az 204)*	++
PR5	-	OR3	+	Control	-
CR1	++	OR4	+		
Endophytic diazotrophs					
CGE2	+	CRE9	+++	ORE13	+
CGE3	+	CRE10	+	CBE8	++
CGE4	++	ORE7	+++	CBE9	++
SSE5	+	ORE8	-	CBE10	++
PRE1	++	ORE9	+	CDE4	++
PRE2	+	ORE10	++	SVE9	++
CRE7	++	ORE11	+	*G.diazotrophicus* (PAL5)*	++
CRE8	++	ORE12	+	Control	-

- no growth (< 0.10 OD) ; + (0.1 to 0.3); ++ (0.3 to 0.5); +++ (>0.5), *standard strain

Table 10. Total nitrogen and ammonia production by diazotrophic isolates from rhizosphere of grass species collected from different physiographic regions

Isolate	Total nitrogen (mg g^{-1} of malate)	Ammonia (mg ml^{-1})
BR1	13.2 (± 0.58)^{abc}	3.0 (± 0.19)^{b}
BR2	1.8 (± 0.36)^{lm}	2.7 (± 0.28)^{bc}
CG1	10.2 (± 0.12)^{de}	3.0 (± 0.24)^{b}
CG3	7.4 (± 0.58)^{gh}	2.7 (± 0.56)^{bc}
CG4	ND	ND
CG5	10.3 (± 0.16)^{de}	1.2 (± 0.13)^{jk}
SS4	9.8 (± 0.35)^{e}	2.3 (± 0.23)^{de}
PR1	9.3 (± 0.35)^{ef}	2.2 (± 0.23)^{def}
PR2	5.1 (± 0.45)^{k}	2.1 (± 0.14)$^{d-g}$
CR1	6.5 (± 0.78)$^{g-k}$	2.2 (± 0.14)^{def}
CR2	12.6 (± 0.23)^{bc}	1.9 (± 0.14)^{eh}
CR3	10.1 (± 0.69)^{de}	1.7 (± 0.12)^{ghi}
DA2	ND	ND
DA5	ND	ND
CB1	7.1 (± 0.45)$^{g-j}$	1.2 (± 0.08)^{jk}
CB2	13.8 (± 0.89)^{ab}	4.2 (± 0.26)^{a}
CB3	10.9 (± 0.98)^{de}	2.2 (± 0.13)^{def}
CB4	11.7 (± 0.42)^{cd}	2.1 (± 0.23)^{ef}
CB5	3.2 (± 0.78)^{l}	1.1 (± 0.09)^{k}
CB6	ND	ND
CB7	2.1 (± 0.20)^{lm}	2.0 (± 0.08)^{ef}
OR1	2.0 (± 0.48)^{lm}	1.9 (± 0.20)$^{e-h}$
OR2	ND	ND
OR3	11.6 (± 0.14)^{cd}	3.0 (± 0.12)^{b}
OR4	2.7 (± 0.18)^{lm}	1.1 (± 0.04) ^{k}
OR5	6.3 (± 0.53)$^{h-k}$	1.4 (± 0.10)^{ijk}
OR6	ND	ND
OR7	9.4 (± 0.10)^{ef}	2.3 (± 0.24)^{de}
OR8	ND	ND
OR9	ND	ND
CD1	14.7 (± 0.12)^{a}	2.7 (± 0.17)^{bc}
CD2	9.3 (± 0.01)^{ef}	2.1 (± 0.16)$^{d-g}$
CD3	6.6 (± 0.14)$^{g-k}$	2.1 (± 0.14)$^{d-g}$
SV1	7.5 (± 0.24)^{gh}	1.9 (± 0.12)$^{e-h}$
SV2	7.2 (± 0.84)^{ghi}	1.2 (± 0.07)^{jk}
SV3	6.4 (± 0.70)$^{g-k}$	2.4 (±0 .15)^{cd}
SV4	ND	ND
A. lipoferum (Az 204)*	5.50 (± 0.60)^{ijk}	1.8 (± 0.13)$^{f-i}$
Control	0.04 (± 0.01)^{o}	0.09 (± 0.01)^{l}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); ND : Not detected; *standard strain.

Table 11. Total nitrogen and ammonia production by endophytic diazotrophs from grass species collected from different physiographic regions.

Isolate	Total nitrogen (mg g^{-1} of malate)	Ammonia (mg ml^{-1})
CGE2	5.1 (± 0.16)^{f}	1.5 (± 0.10)^{hi}
CGE3	10.5 (± 0.65)^{abc}	3.0 (± 0.19)^{b}
CGE4	7.3 (± 0.11)^{e}	1.4 (± 0.14)^{i}
SSE5	2.3 (± 0.69)^{g}	1.1 (± 0.12)^{j}
PRE1	5.1(± 0.45)^{f}	1.1 (±0.08)^{j}
PRE2	9.0 (± 0.20)^{cde}	1.7 (± 0.14)^{fg}
CRE7	10.4 (± 0.46)^{abc}	1.7 (± 0.07)^{fg}
CRE8	2.2 (± 0.12)^{g}	1.8 (± 0.11)^{ef}
CRE9	11.7 (± 0.23)^{a}	3.0 (± 0.19)^{b}
CRE10	11.0 (± 0.85)^{ab}	5.4 (± 0.37)^{a}
ORE7	9.8 (± 0.60)^{bcd}	1.9 (± 0.12)^{e}
ORE9	11.4 (± 0.19)^{ab}	2.3 (± 0.14)^{d}
ORE10	5.4 (± 0.18)^{f}	1.2 (± 0.07)^{gh}
ORE11	ND	ND
ORE12	8.3 (± 0.50)^{de}	2.6 (± 0.06)^{c}
ORE13	1.2 (± 0.40)^{h}	ND
CBE8	7.6 (± 0.85)^{e}	1.2 (± 0.05)^{j}
CBE9	2.5 (± 0.51)^{g}	1.9 (± 0.20)^{e}
CBE10	5.7 (± 0.10)^{f}	1.6 (± 0.12)^{gh}
CDE4	ND	ND
SVE9	5.7 (± 0.88)^{f}	1.4 (± 0.11)^{i}
A. lipoferum (Az 204)*	5.5 (± 0.60)^{f}	1.8 (± 0.13)^{ef}
Control	0.04 (± 0.01)^{i}	0.03 (± 0.01)^{k}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$);ND : Not detected; * standard strain.

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Table 12. Nitrogenase activity of diazotrophic isolates from rhizosphere of grass species collected from different physiographic regions

Isolate	Nitrogenase activity (n moles of ethylene mg^{-1} of protein h^{-1})
BR1	84.45 (± 3.98)^{k}
BR2	ND
CG1	107.37 (± 14.35)^{g}
CG3	99.58 (± 6.41)^{i}
CG5	120.5 (± 21.65)^{f}
SS4	133.0 (±9.23)^{c}
PR1	ND
PR2	ND
CR1	ND
CR2	131.79(± 14.46)^{d}
CR3	89.20 (± 5.43)^{k}
CB1	ND
CB2	99.58 (± 4.89)^{i}
CB3	75.45 (± 4.29)^{l}
CB4	127.7 (± 14.40)^{e}
CB5	ND
CB7	ND
OR1	ND
OR3	172.25 (± 13.95)^{a}
OR4	ND
OR5	50.83 (± 3.28)^{m}
OR7	131.57 (± 12.57)^{d}
CD1	151.58 (± 16.27)^{b}
CD2	95.91 (± 5.41)^{j}
CD3	ND
SV1	108.4 (± 8.45)^{g}
SV2	ND
SV3	ND
A. lipoferum (Az 204)*	48.29 (± 1.03)^{n}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain; ND- not detected

Table 13. Nitrogenase activity of endophytic diazotrophs in grass species collected from different physiographic regions

Isolate	Nitrogenase activity (n moles of ethylene mg^{-1} of protein h^{-1})
CGE2	ND
CGE3	171.45 (± 11.80)[a]
CGE3	ND
SSE5	ND
PRE1	ND
PRE2	91.33 (± 6.51)[f]
CRE8	ND
CRE9	163.45 (± 21.56)[b]
CRE10	155.35 (± 15.41)[c]
CBE8	ND
CBE9	87.33 (± 4.14)[g]
CBE10	ND
ORE7	116.16 (± 20.8)[e]
ORE8	ND
ORE9	125.79 (± 23.24)[d]
ORE10	ND
ORE11	ND
ORE12	ND
ORE13	ND
CDE4	ND
SVE9	83.37 (± 4.67)[h]
G. diazotropicus (PAL5) *	47.0 (± 3.41)[h]

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); ND : not detected; *standard strain

diazotrophic isolates ranged from 48.29 ± 1.03 to 172.25 ±1.95 n moles ethylene mg^{-1} protein h^{-1}. The highest nitrogenase activity was exhibited by isolate OR3 (172.25 ±13.95 n moles ethylene mg^{-1} protein h^{-1}) followed by CD1 (151.58 ±13.27 n moles ethylene mg^{-1} protein h^{-1}). The standard culture *A. lipoferum* (Az 204) showed comparatively lower ARA activity (48.29 ±1.03 n moles ethylene mg^{-1} protein h^{-1}) than the other isolates (Table 12).

From the endophytic diazotrophs, highest ARA activity was found in CGE3 (171.45 ±11.80 n moles ethylene mg^{-1} protein h^{-1}). The standard culture *G.diazotrophicus* showed the least nitrogenase activity of 47.0 ±1.41 n moles ethylene mg^{-1} protein h^{-1}(Table 13).

Based on the nitrogenase activity, 16 isolates from rhizosphere and 8 isolates from endophytes were selected and used for further studies.

4.5. Morphological characterization of diazotrophic isolates from selected grass species

The selected both rhizosphere and endophytic diazotrophic bacterial colonies were characterized by cell shape, morphology, Gram reaction (Table. 14). Among the sixteen isolates, four were Gram positive and remaining isolates were Gram negative. In endophytes three were Gram positive and the rest were Gram negative. In general all the diazotrophs were rod shaped cells of varying length. The colony morphology varied from small, round, circular, raised or irregular. The isolates exhibited varied colony colours *viz.*, white, off-white, dull white, yellow, orange and cream.

4.6. Metabolic diversity of diazotrophs isolated from selected grass species

4.6.1. Differential carbon substrate utilization by diazotrophs

The growth of diazotrophic isolates in modified nitrogen free medium supplemented with different carbon substrates was recorded as presence or absence of growth after seven days of incubation (Table 15). Differences were observed in the carbon substrate utilization profile among the isolates. The dendrograms showing the relatedness of 24 rhizosphere and endophytic diazotrophs are depicted in Fig.5. In dendrogram, the Jaccard's similarity coefficient (≥ 0.75) clustered the both rhizospheric and endophytic diazotrophs into 7 different groups. The results revealed that

Table 14. Morphological characterization of diazotrophic isolates from grass species collected from different physiographic regions

Isolate	Colony Morphology	Colony Colour	Cell Shape	Gram reaction
Rhizosphere diazotrophs				
BR1	irregular	creamy white	rod	-
CG1	circular	creamy white	rod	-
CG3	irregular	cream	rod	-
CG5	round	creamy white	rod	+
SS4	irregular	dull white	rod	+
CR2	round	white	rod	-
CR3	small round	creamy white	rod	-
CB2	round	cream	rod	-
CB3	circular	creamy white	rod	+
CB4	round	orange	rod	-
CD1	circular	dull white	rod	-
CD2	small round	dull white	rod	+
OR3	small round	creamy white	rod	-
OR5	irregular	creamy white	rod	-
OR7	circular	creamy white	rod	-
SV1	circular	white	rod	-
Endophytic diazotrophs				
CGE3	circular	creamy white	curved rods	-
PRE2	circular	pale yellow	rod	-
CRE9	circular	cream	rods	+
CRE10	irregular	creamy white	rod	-
CBE9	circular	creamy white	rod	+
ORE7	round	creamy white	rod	-
ORE9	circular	dull white	rod	-
SVE9	irregular	white	rod	+

+ Gram positive; - Gram negative

Table 15. Differential carbon substrate utilization pattern of diazotrophic isolates of grass species collected from different physiographic regions

Isolate	Carbon source[a]											
	D-Glucose	D-Xylose	Fructose	Mannitol	Amylose	Betaine	Citrate	Cellulose	Starch	Glycerol	Tartrate	Acetate
Rhizosphere diazotrophs												
CG1	+	-	-	+	+	-	+	+	+	+	+	+
BR1	+	+	-	+	+	-	+	+	+	+	+	+
CG3	+	+	-	+	+	-	+	+	+	+	+	-
CG5	+	+	-	+	+	+	-	+	+	+	+	+
CD1	+	-	+	+	-	-	+	+	+	-	-	+
CD2	+	+	-	+	+	-	-	+	+	+	+	+
CB2	+	+	-	-	+	+	+	+	+	+	-	+
CB3	+	+	-	+	-	+	+	+	-	-	-	-
CB4	+	-	-	+	-	+	+	+	+	+	-	+
CR2	+	-	-	+	+	+	+	+	+	-	-	+
CR3	+	+	-	+	-	+	+	+	-	-	+	-
SV1	+	+	+	+	+	-	+	+	+	-	+	-
SS4	+	-	+	+	-	-	+	+	-	-	+	-
OR3	+	+	-	-	+	+	+	+	+	-	-	+
OR5	+	-	-	+	+	-	+	+	+	+	+	+
OR7	+	-	+	+	-	-	+	+	-	-	+	+
Endophytic diazotrophs												
CGE3	+	+	-	+	+	-	+	+	+	+	-	-
PRE2		_	-	+	+	-	+	+	+	+	+	-
ORE7	+	+	-	+	-	+	+	+	-	-	-	+
ORE9	+	+	-	+	-	-	+	-	+	+	+	-
CBE9	+	+	-	+	-	+	+	+	-	-	-	+
CRE9	+	-	+	+	-	-	+	+	-	-	-	+
CRE10	+	+	-	+	+	-	+	+	+	+	+	+
SVE9	+	+	+	+	+	-	+	+	+	-	-	+

[a] Carbon substrates were supplemented in modified N free medium at 1% (w/v);+ Presence of growth; - Absence of growth

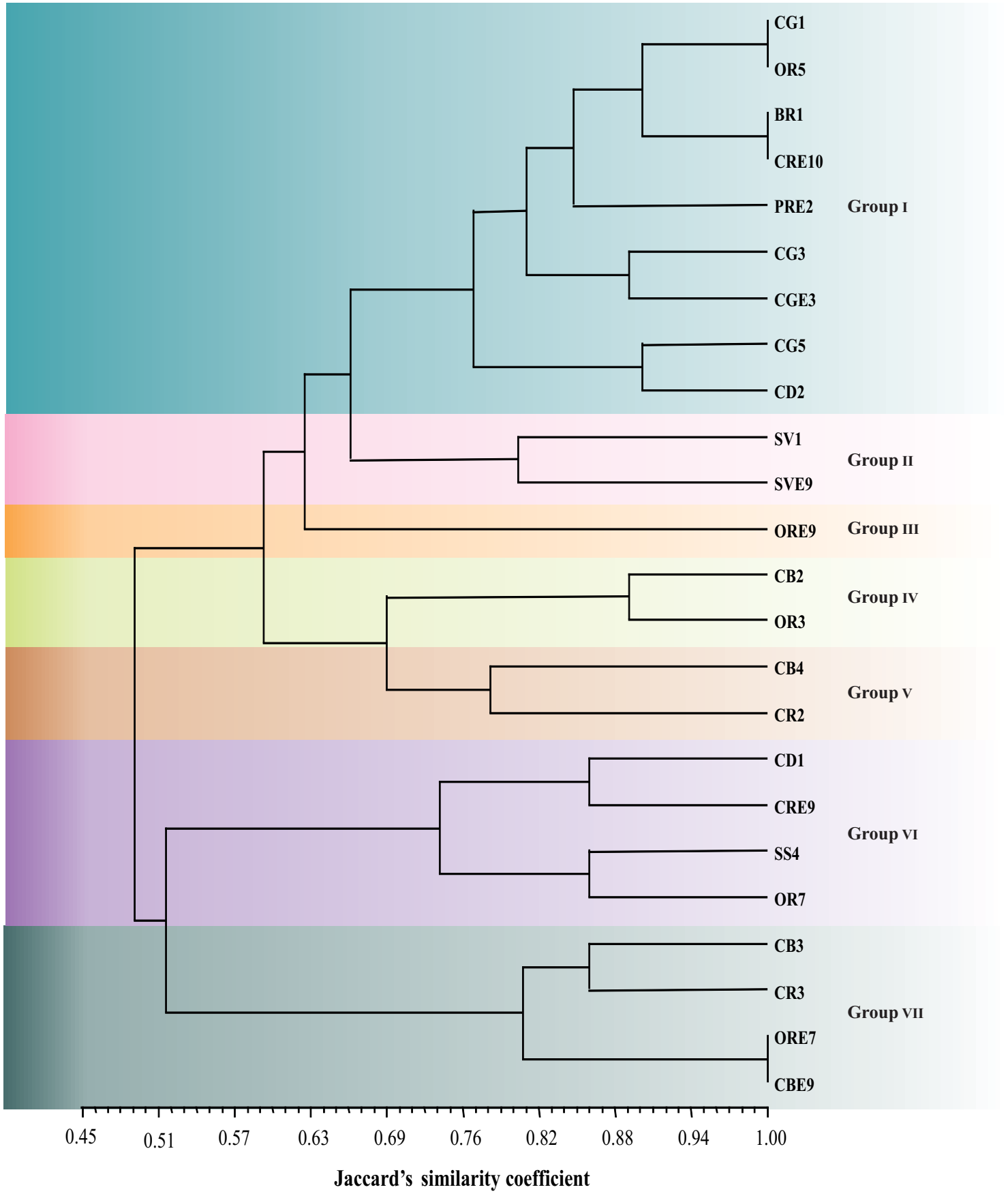

Fig. 5. Dendrogram showing relatedness of diazotrophic isolates from grass species of different physiographic region using differential carbon substrate utilization profiling. The binary data showing presence or absence of growth in different carbon substrates were used for clustering the isolates by Jaccard's similarity index. The coefficient ≥ 0.75 was used to group the isolates into different groups.

group I comprised of nine isolates. Group II, IV, and V comprised of each 2 isolates. One isolate ORE9 formed group III and the Group VI consists of CRE9, SS4, CD1 and OR7 and group VII comprised of four isolates.

4.6.2. Intrinsic antibiotic resistance (IAR) of diazotrophs

The presence or absence of inhibition zone around the antibiotic discs was observed to validate the susceptibility or resistance of the isolates to 12 different antibiotics (Table 16). Using the similarity index ($\geq$ 0.75), clustering of the isolates was calculated and represented as dendrogram (Fig. 6). In dendrogram, the Jaccard's similarity coefficient ($\geq$ 0.75) clustered the rhizospheric and endophytic diazotrophs into 7 different groups. The results showed that group I comprised of ten isolates and group III clustered to seven isolates. The group II and VII consist of each two isolates only. The group IV, V and VI have only one isolate.

4.7. Identification of rhizosphere and endophytic diazotrophic isolates by 16S rRNA gene sequence homology and phylogeny construction

The total genomic DNA of all the 24 isolates from rhizosphere and endophytic were extracted to amplify the 16S rRNA (Plate 2). High concentration of good quality genomic DNA with A260 / A280 value ranging between 1.8 and 2.0 was obtained. In order to gain insights about the bacterial identity, the full length DNA amplification and sequencing of 16S rRNA gene was performed (Plate 3). The 16S rRNA gene of all the 24 isolates was amplified using universal primers FD1 and RP2. All amplified products produced a single band with approximately 1500 bp length and the differences among them were not visible in 1 per cent agarose gel.

Comparative BLAST analyses which include the closest species, and per cent homology of full length 16S rRNA revealed the presence of diversity of Gamma proteobacteria and Firmicutes (Table 17). The isolates CR2, CR3 SV1 showed close similarity to *K. pneumonia* and the isolates CG1, CB4, OR7 found close similar to *Klebsiella* sp. The isolates BR1, CG3 and ORE7 were found to be closely similar to *Enterobacter* sp. The isolate SVE9 and CD1 belong to *E. sacchari* and *S. marcescens* respectively. The isolates CB2, OR3 and CRE9 showed high resemblance to *Serratia* sp. The isolate CB3 was closely resemble to *B. subtilis*. The isolates CG5 and CD2 which

Table 16. Intrinsic antibiotic resistance (IAR) profile of diazotrophic isolates of grass species collected from different physiographic regions

Isolates	Antibiotics											
	A	At	Cj	Au	Ce	Cs	Cu	E	P	Cf	Cw	Cq
Rhizosphere diazotrophs												
BR1	-	-	+	-	+	+	+	-	-	+	-	-
CG1	-	-	+	-	+	+	-	-	-	+	-	-
CG3	-	-	+	-	+	+	-	-	-	+	+	-
CG5	-	-	+	-	+	+	+	-	-	+	-	-
SS4	-	-	+	+	+	+	+	-	-	+	+	-
CR2	-	-	+	+	+	+	+	-	-	+	-	-
CR3	-	-	+	+	+	+	+	-	-	+	-	-
CB2	-	-	+	-	+	+	+	-	-	+	-	-
CB3	-	-	+	-	+	+	+	-	-	+	-	-
CB4	-	-	-	-	+	+	+	-	-	+	+	-
OR3	-	-	+	+	+	+	+	-	-	+	+	-
OR5	-	-	+	+	+	+	+	-	-	+	+	-
OR7	-	-	+	+	+	+	+	-	-	+	+	-
CD1	-	-	+	-	+	+	+	-	-	+	-	-
CD2	-	-	+	-	+	+	+	-	-	+	-	-
SV1	-	-	-	-	+	+	+	-	-	+	+	-
Endophytic diazotrophs												
CGE3	-	-	+	-	+	+	+	-	-	+	-	-
PRE2	-	-	+	-	+	+	+	-	-	-	-	-
ORE7	-	-	+	-	+	+	+	-	-	-	-	-
ORE9	-	-	+	-	+	+	+	+	-	+	+	-
CBE9	-	-	+	+	+	+	+	-	-	+	+	+
CRE9	-	-	+	+	+	+	+	-	-	+	+	+
CRE10	-	-	+	+	+	+	+	-	-	+	+	-
SVE9	-	-	+	+	+	+	+	-	-	+	-	-

+ Presence of growth; - Absence of growth

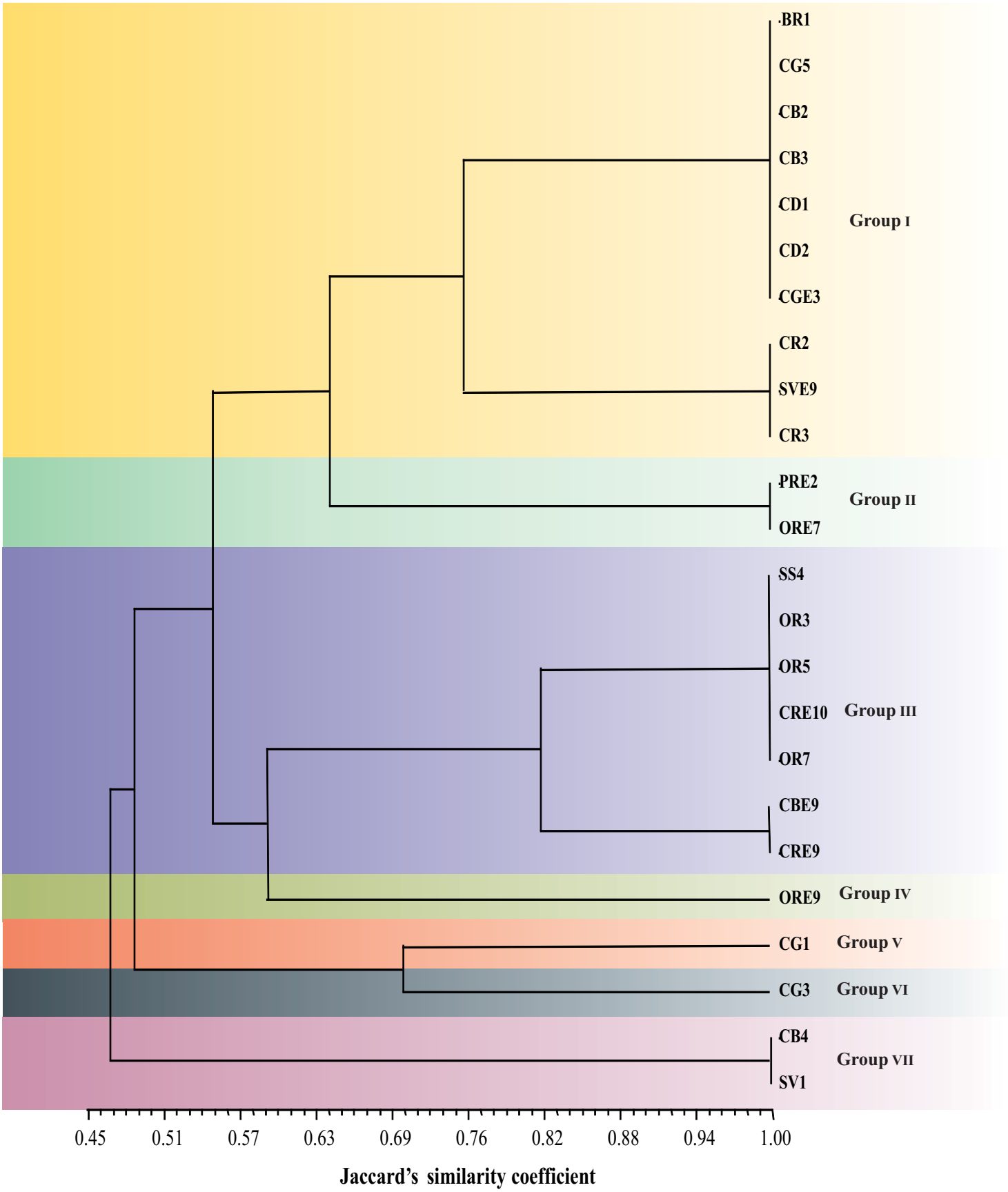

Fig. 6. Dendrogram showing relatedness of diazotrophic isolates from grass species of different physiographic region using intrinsic antibiotic resistance (IAR). The binary data showing presence or absence of growth in different carbon substrates were used for clustering the isolates by Jaccard's similarity index. The coefficient ≥0.75 was used to group the isolates into different groups.

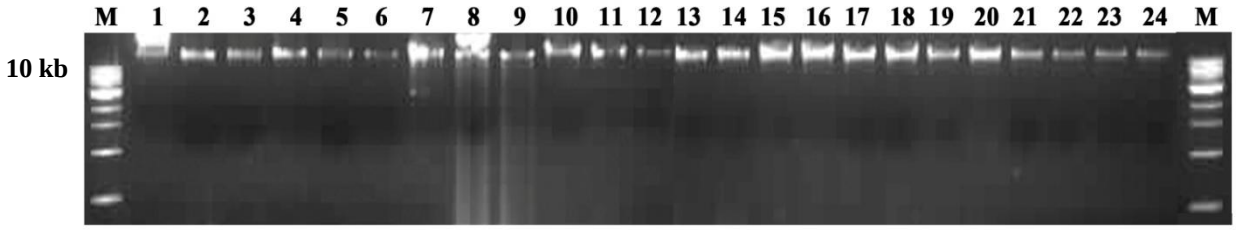

Plate 2. Agarose gel electrophoresis of genomic DNA extracted from diazotrophs isolated from grass species. M- 1kb DNA ladder; 1 to 16- Rhizosphere diazotrophic isolates; 17-24- Endophytic diazotrophs.

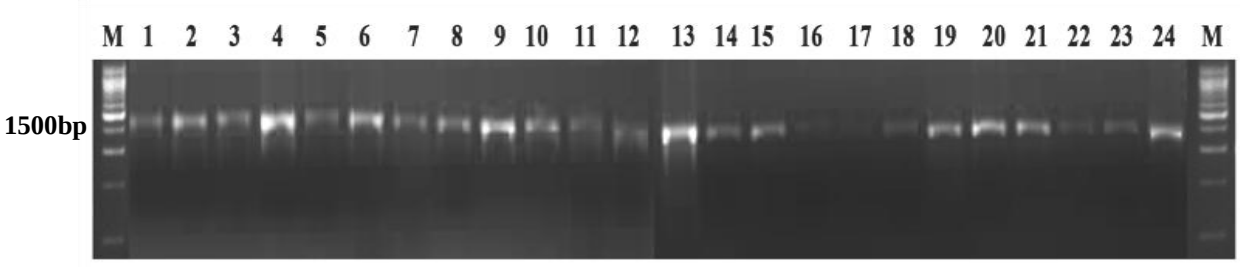

Plate 3. Agarose gel electrophoresis of amplicons of 16S rRNA gene from diazotrophic isolates from grass species. M- 1kb DNA ladder; 1 to 16- Rhizosphere diazotrophic isolates; 17-24- Endophytic diazotrophs.

similar to *Stenotrophomonas* sp. and *Bacillus* sp. respectively. The isolates PRE2 and ORE9 closely related to *Pantoea* sp. and *P. agglomerans* respectively. The percentage similarity of all the isolates to the closest sequence in the Genbank ranged from 94-100 per cent except the isolates CGE3 (*Pseudomonas* sp.) and CRE 10 (*Pseudomonas* sp.) were found 85 and 86% homology respectively. The 16S rRNA gene sequences from the diazotrophic isolates and the available16S rRNA gene sequences from NCBI were aligned and used for construction of phylogenetic tree (Fig. 7).

4.8. Screening of diazotrophic isolates for the presence of gene (*nifH*)

The diazotrophic isolates from selected grass species were screened for the presence of *nifH* gene by detection of partial amplification of *nifH* gene. The universal primers nifHb1 and CDHPnif723R which could amplify 550 bp, were used for screening the isolates by PCR amplification. The agarose gel of PCR amplification of *nifH* of all the 24 isolates are presented in Plate 4. The *nifH* amplification could be clearly detected in 10 isolates from rhizosphere *viz*., *Klebsiella* sp.(CG1) *Enterobacter* sp.(CG3), *Bacillus* sp. (CG5), *K. pneumoniae* (CR2), *K. pneumoniae* (CR3), *Serratia* sp.(CB2), *B. subtilis* (CB3), *S. marcescens* (CD1), *K. pneumonia* (SV1), *Serratia* sp.(OR3) and 5 isolates from endophytes *Pseudomonas* sp. (CGE3), *Serratia* sp. (CRE9), *Pseudomonas* sp. (CRE10), *Enterobacter* sp.(ORE7) and *P. agglomerans* (ORE9).

The amplified *nifH* products were further digested with *HaeIII* enzyme and the banding pattern were observed (Plate 5). From the results it was clearly showed that, all diazotrophic isolates having the similar banding patterns. Since there is no difference of *nifH* gene among the diazotrophic isolates.

4.9. Plant growth promoting characteristics of diazotrophs isolated from different grass species

4.9.1. Production of plant growth promoting substances

All the rhizosphere and endophytic diazotrophic isolates gave positive result with regard to IAA production (Fig. 8A and 8B). Among the rhizosphere diazotrophs, the maximum amount of IAA was produced by *S. marcescens* (CD1) (30.80 ±1.14 µg mg^{-1} protein) followed by *K. pneumoniae* (CR2) (25.60 ± 2.23 µg mg^{-1} protein). In endophytic

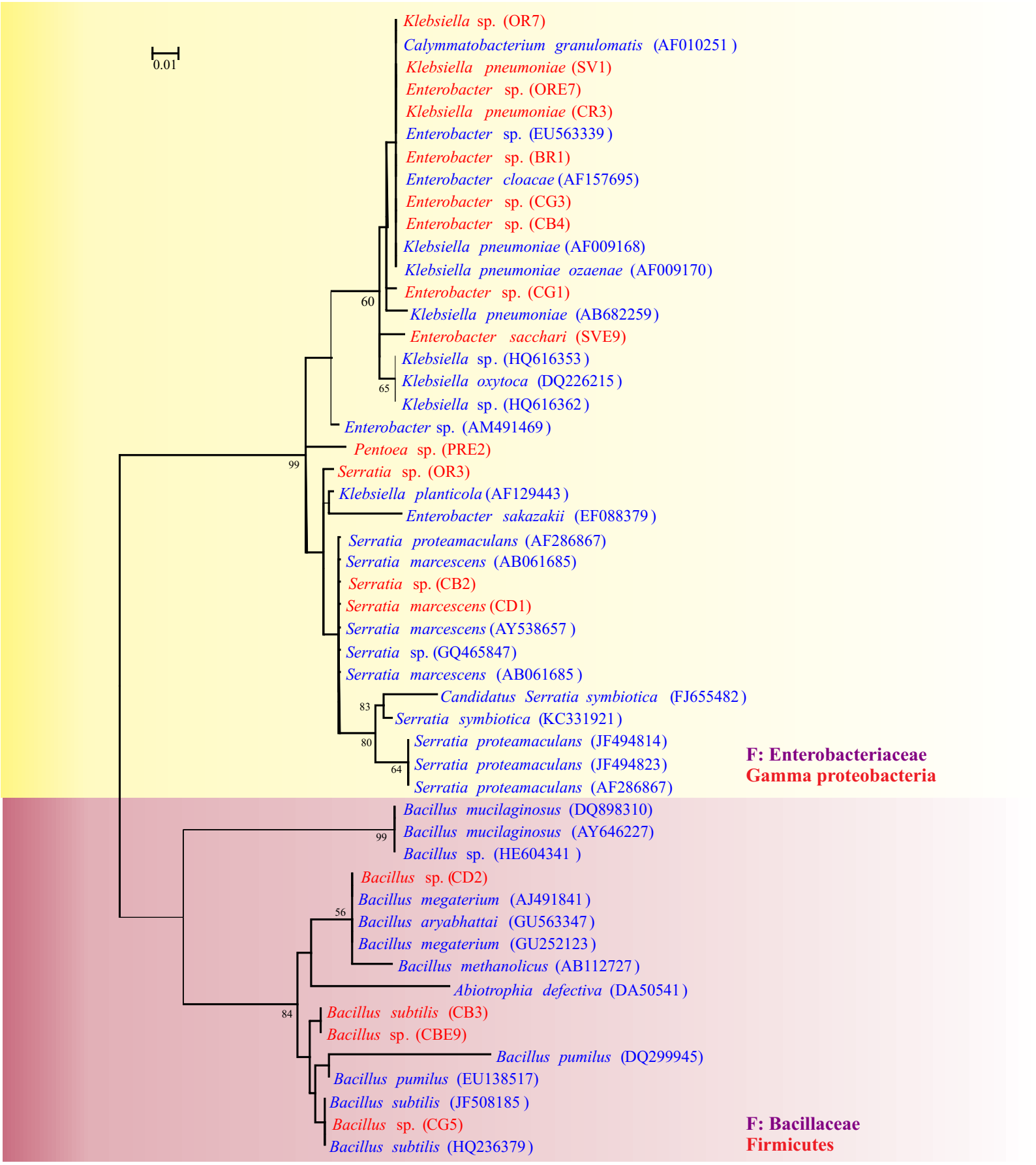

Fig. 7. Neibhour-joining phylogenetic tree based on partial 16S rRNA gene of diazotrophic isolates showing similarity with bacteria from different groups (Accession numbers are in parentheses). The percentage of 1000 bootstrap replicates are shown at the nodes when at least 50%. The scale bar indicates one changes per 100 bp nucleotides.

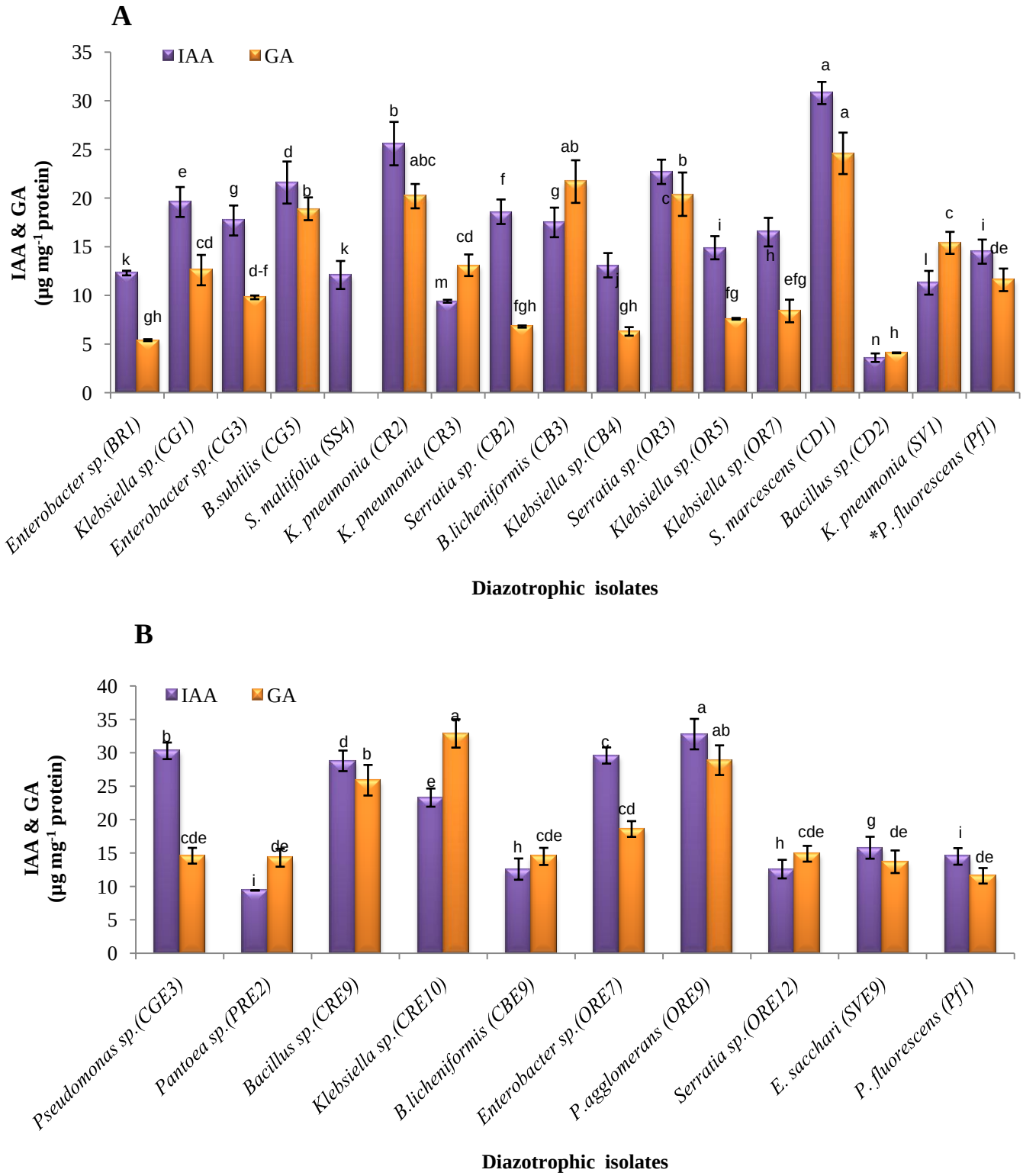

Fig. 8. Indole acetic acid and gibberellic acid production by rhizosphere diazotrophs (A) and endophytes (B) of grass species. Data are presented as mean ± SE (n=3); For each panel, different letters indicate significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

Table 17. Authentication of diazotrophic isolates from grass species of different physiographic regions by 16S rRNA gene sequence homology

Isolate	Grass species	Species homology[a]	Percent homology[b]	GenBank Accession No
Rhizosphere diazotrophs				
BR1	*B. repens*	*Enterobacter* sp.	99	KF906826
CG1	*C. glaucus*	*Klebsiella* sp.	94	KF906827
CG3	*C. glaucus*	*Enterobacter* sp.	98	KF906828
CG5	*C. glaucus*	*Bacillus* sp.	99	KF906830
SS4	*S. spontaneum*	*Stenotrophomonas* sp.	98	NS
CR2	*C. rotundus*	*Klebsiella pneumoniae*	98	NS
CR3	*C. rotundus*	*Klebsiella pneumoniae*	99	KF906829
CB2	*C. barbata*	*Serratia* sp.	99	KF906831
CB3	*C. barbata*	*Bacillus subtilis*	98	NS
CB4	*C. barbata*	*Klebsiella* sp.	98	NS
OR3	*O. rufipogon*	*Serratia* sp.	96	KF906832
OR5	*O. rufipogon*	*Staphylococcus saprophyticus*	99	KF906833
OR7	*O. rufipogon*	*Klebsiella* sp.	98	KF906834
CD1	*C. dactylon*	*Serratia marcescens*	97	KF906835
CD2	*C. dactylon*	*Bacillus* sp.	99	NS
SV1	*S.verticillata*	*Klebsiella pneumoniae*	99	KF906836
Endophytic diazotrophs				
CGE3	*C. glaucus*	*Pseudomonas* sp.	85	NS
PRE2	*P. repens*	*Pantoea* sp.	98	KF906837
CRE9	*C. rotundus*	*Serratia* sp.	99	KF906838
CRE10	*C. rotundus*	*Pseudomonas* sp.	86	KF906839
CBE9	*C. barbata*	*Bacillus* sp.	99	KF906840
ORE7	*O. rufipogon*	*Enterobacter* sp.	97	KF906841
ORE9	*O. rufipogon*	*Pantoea agglomerans*	94	KF906842
SVE9	*S.vetricillata*	*Enterobacter sacchari*	99	KF906843

[a] Species identified based on the 16S rRNA gene sequence similarity by BLAST ; [b] Per cent similarity of the isolate's sequence in BLAST result; NS-Sequence not submitted

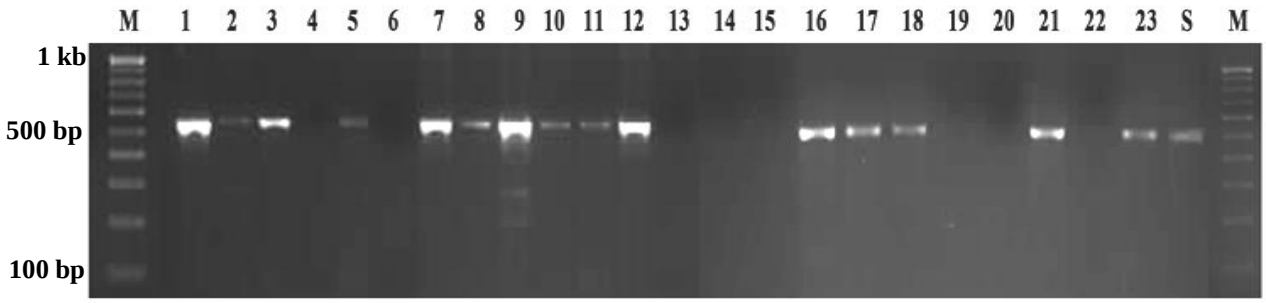

Plate 4. Agarose gel electrophoresis showing *nifH* gene amplified from diazotrophs isolated from selected grass species. M- 100 bp DNA ladder; 1- *Klebsiella* sp. (CG1), 2- *Enterobacter* sp. (CG3), 3- *B. subtilis* (CG5), 4- *Stenotrophomonas* sp. (SS4), 5- *K. pneumoniae* (CR2), 6- *Klebsiella* sp. (CB4), 7- *K. pneumoniae* (CR3), 8- *Serratia* sp.(CB2), 9 *B. licheniformis* (CB3), 10-*Serratia* sp. (OR3), 11- *S. marcescens* (CD1), 12- *K. pneumoniae* (SV1), 13- *Klebsiella* sp. (Or5), 14- *Klebsiella* sp. (OR7), 15- *Bacillus* sp. (CD2), 16- *Pseudomonas* sp.(CGE3), 17-*Serratia* sp. (CRE9), 18 - *Pseudomonas* sp.(CRE10), 19 - *Enterobacter* sp.(BR1), 20- *B.licheniformis* (CBE9), 21- *Enterobacter* sp (ORE7), 22- *E. sacchari* (SVE9), 23- *P. agglomerans* (ORE9), S- Az 204-*A. lipoferum*.

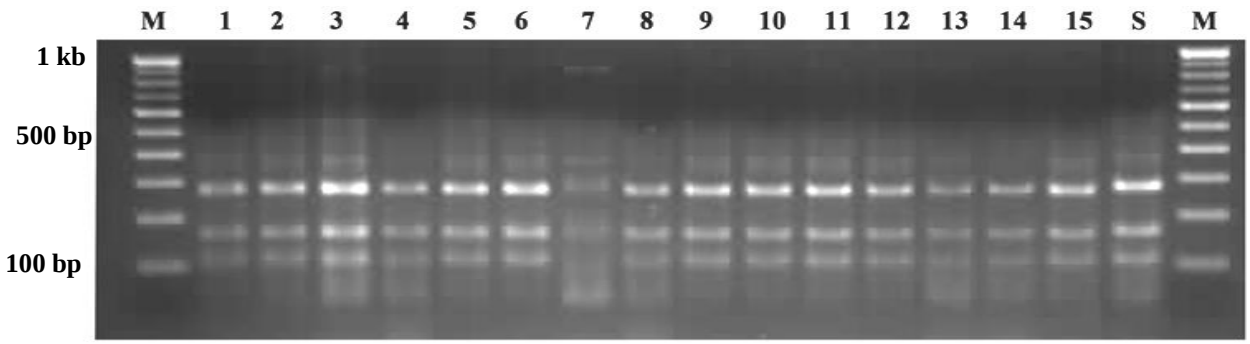

Plate 5. ARDRA profile of *nifH* gene of diazotrophs isolated from selected grass species. The amplified 16S rRNA gene from the isolates were digested with *Hae III* and resolved in 1.5% agorose. M - 100 bp DNA ladder; 1- *Klebsiella* sp. (CG1), 2-*Enterobacter* sp. (CG3), 3-*B.subtilis* (CG5), 4- *K. pneumoniae* (CR2), 5-K. *pneumoniae* (CR3), 6-*Serratia* sp.(CB2), 7- *B. licheniformis* (CB3), 8-*Serratia* sp. (OR3), 9-S. *marcescens* (CD1), 10-K. pneumoniae (SV1), 11-*Pseudomonas* sp. (CGE3), 12-*Serratia* sp. (CRE9), 13-*Pseudomonas* sp.(CRE10), 14-*Enterobacter* sp (ORE7), 15- *P. agglomerans* (ORE9), S-Az 204-*A. lipoferum.*

diazotrophs, the maximum amount of IAA was produced by *P. agglomerans* (ORE9) (32.8 ± 2.28 µg mg^{-1} protein).

All but one isolate from rhizosphere produced GA where, the highest amount was produced by *S. marcescens* (CD1) (24.60 ± 2.13 µg mg^{-1} protein) and the least produced by *Bacillus* sp. (CD2) (4.10 ± 0.04) µg mg^{-1} protein. Among the endophytic diazotrophs, the highest amount of GA was produced by *Pseudomonas* sp. (CRE10) (32.9 ± 2.12µg ml^{-1}) (Fig. 8A and 8B).

4.9.2. ACC deaminase activity

Among the rhizosphere diazotrophic isolates, seven were able to produce ACC deaminase where the isolate *K. pneumoniae* (CR2) registered the highest activity of 92.5 ± 8.12 n moles α-ketobutyrate mg^{-1} h^{-1}. The least ACC deaminase activity was produced by standard culture *P. fluorescens* (Pf1) of 66.9 ± 4.61 n moles α-ketobutyrate mg $^{-1}$ h^{-1} (Table 18).

Among the endophytic diazotrophs, five were able show the ACC deaminse activity. The isolate *P.agglomerans* (ORE9) recorded maximum ACC deaminse activity (123.5 ± 8.17 n moles of α-ketobutyrate mg $^{-1}$ h^{-1}) followed by *Enterobacter* sp. (ORE7) (105.8 ±9.56 n moles of α-ketobutyrate mg $^{-1}$ h^{-1}) (Table 18).

4.9.3. Siderophore and HCN production

All the rhizosphere and endophytic diazotrophic isolates were able to produce siderophore. The maximum siderophore production was recorded in *S. marcescens* (CD1) (66.30 ±3.21 µg mg^{-1} protein) followed by *K. pneumoniae* (CR2) (54.6 ± 2.14 µg mg^{-1} protein). Among the endophytic diazotrophs, the maximum (35.5 ± 1.22 µg mg^{-1} protein) siderophore was produced by *Pseudomonas* sp. (CRE10) followed by *Bacillus* sp. (CBE9) (28.5 ± 0.30 µg mg^{-1} protein) (Table 18).

Seven isolates from rhizosphere and five isolates from endophytes were reported positive results for HCN production which was further quantified. The result showed that the isolate *S. marcescens* (CD1) recorded higher hydrogen cyanide production of 68.70±1.13 of µg mg^{-1} protein followed by *Serratia* sp. (CB2) and *K. pneumoniae* (CR2) that showed 62.4 ±1.58 and 60.80 ±1.18 µg mg $^{-1}$ protein respectively (Table 18). Among the endophytes, maximum HCN production was recorded in *Pseudomonas* sp. (CRE10)

Table 18. Plant growth promoting activities of diazotrophic isolates from rhizosphere of grass species

Isolate	ACC deaminse (nmoles of α-ketobutyrate mg^{-1} protein h^{-1})	Siderophore (Catechol type) ($\mu g\ mg^{-1}$ protein)	HCN ($\mu g\ mg^{-1}$ protein)
Rhizosphere diazotrophs			
Enterobacter sp.(BR1)	ND	20.9 (± 1.15)^{fg}	ND
Klebsiella sp.(CG1)	ND	13.5 (± 1.18) ^{ghi}	30.6 (± 1.56)^{f}
Enterobacter sp.(CG3)	ND	22.4 (± 1.10)^{de}	35.4 (± 1.24)^{e}
Bacillus sp. (CG5)	74.3 (± 7.98)^{c}	38.4 (±1.19)^{d}	44.5 (± 1.37)^{cde}
Stenotrophomonas sp. (SS4)	ND	18.9 (± 1.24)^{fg}	ND
K. pneumoniae (CR2)	92.5 (± 8.12)^{a}	54.6 (± 2.14)^{b}	60.8 (±1.18)^{b}
K. pneumoniae (CR3)	ND	13.5 (± 1.13)^{ghi}	ND
Serratia sp. (CB2)	83.9 (± 4.96)^{ab}	23.5 (± 2.11)^{de}	62.4 (± 1.58)^{b}
B.subtilis (CB3)	87.9 (± 3.29)^{ab}	18.4 (± 1.70)^{fgh}	ND
Klebsiella sp.(CB4)	ND	20.4 (± 2.24)^{fg}	ND
Serratia sp.(OR3)	76.8 (± 2.98)^{c}	66.3 (± 3.21)^{a}	68.7 (± 1.13)^{a}
S. saprophyticus (OR5)	ND	20.9 (±2.26)^{fg}	ND
Klebsiella sp.(OR7)	ND	49.3 (± 2.27)^{c}	ND
S. marcescens (CD1)	85.3 (± 6.78)^{ab}	22.4 (±1.24)^{de}	40.5 (±1.19)^{de}
Bacillus sp.(CD2)	86.9 (± 9.45)^{ab}	5.6 (± 0.13)^{k}	ND
K. pneumoniae (SV1)	ND	9.8 (± 1.10)^{hi}	ND
Endophytic diazotrophs			
Pseudomonas sp.(CGE3)	ND	17.3 (± 1.24)^{d}	ND
Pantoea sp. (PRE2)	ND	16.8 (± 1.16)^{d}	ND
Serratia sp. (CRE9)	95.8 (± 8.56)^{c}	19.2 (± 1.25)^{cd}	43.8 (± 2.54)^{c}
Pseudomonas sp.(CRE10)	67.9 (± 3.10)^{d}	35.5 (± 1.22)^{a}	68.4 (± 6.58)^{a}
Bacillus sp. (CBE9)	ND	28.5 (± 1.30)^{b}	ND
Enterobacter sp.(ORE7)	105.8 (± 9.56)^{b}	16.3 (± 1.64)^{d}	54.9 (± 5.18)^{b}
P.agglomerans (ORE9)	123.5 (± 8.17)^{a}	17.5 (± 1.32)^{d}	33.8 (± 2.54)^{d}
E. sacchari (SVE9)	63.5 (± 4.04)^{d}	16.4 (± 1.24)^{d}	43.4 (± 1.19)^{c}
P. fluorescens (Pf1) *	66.9 (± 4.61)^{d}	29.4 (± 2.18)^{b}	33.3 (± 1.34)^{d}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$);*standard strain; ND- not detected

(68.4±6.58 µg mg^{-1} protein) followed by *Enterobacter* sp. (ORE7) (54.9±1.18 µg mg^{-1} protein) (Table 18).

4.9.4. Antagonistic activity

The antagonistic activity of all the diazotrophic isolates against 4 plant pathogenic organism *viz.*, bacterial leaf blight (*Xanthomonas oryzae*), sheath blight (*Rhizoctonia solani),* blast (*Pyricularia oryzae*) and sheath rot (*Sarocladium oryzae*) were evaluated. Among the 16 isolates, 1 isolate *Enterobacter* sp. (BR1) inhibited the *X. oryzae,* 11 isolates inhibited the *R. solani,* 9 were found to inhibite the *S. oryzae* and 9 isolates were effective against *P. oryzae.* Four isolates were inhibited three pathogens except *P. oryzae* (Table 19).

Among the endophytes, only 7 isolates were effective against *R. solani* and 6 isolates were able to inhibit the *S. oryzae* and 4 were found effective against *P. oryzae*. None of the endophytic isolates have antagonistic activity against *X. oryzae* (Table 19).

4.10. Mineral solubilization by diazotrophic isolates from selected grass species

4.10.1. *In vitro* phosphorus solubilizing potential of diazotrophic isolates

In the qualitative assay, all the sixteen rhizosphere diazotrophs were found to be positive where, *Serratia* sp. (CB2) and *S. marcescens* (CD1) exhibited maximum solubilization efficiency of 250 and 233 per cent respectively (Table 20). The solubilizing efficiency of endophytic diazotrophs was ranged from 75 to 250 per cent (Table 21) where *Serratia* sp. (CRE9) exhibited maximum solubilization efficiency (250 ± 2.58 per cent).

Influence of phosphorus solubilizing organisms on the pH titrable acidity, available phosphorus and phosphatase enzyme production was studied and the results are given in Table 22 and 23. In general, pH of the medium was decreased with growth of all phosphorus solubilizing isolates. Among the sixteen rhizosphere isolates, not much variation pH reduction was observed but for the titrable acidity increased due to the growth of phosphorus solubilizing organisms. Maximum titrable acidity of 3.4 ±0.01 per cent was found with *S. marcescens* (CD1). The amount of available phosphorus was significantly higher in *Klebsiella* sp. (OR7) (0.96 ± 0.09 µg ml^{-1}) compared to the other isolates. The phosphatase activity was higher in *Staphylococcus saprophyticus* (OR5) (12.9 ±0.10 µg of PNP released ml^{-1} day^{-1}) followed by *Klebsiella* sp. (OR7) (10.90 ± 0.29 µg of PNP released ml^{-1} day^{-1}) (Table 22).

Table 19. Antagonistic activity of diazotrophic isolates from grasses species against rice plant pathogens

Isolate	Rice plant pathogens			
	X. oryzae	*R. solani*	*S. oryzae*	*P. oryzae*
Rhizosphere diazotrophs				
Enterobacter sp.(BR1)	+	-	-	-
Klebsiella sp.(CG1)	-	+	+	-
Enterobacter sp.(CG3)	-	+	-	-
Bacillus sp. (CG5)	-	+	-	-
Stenotrophomonas sp. (SS4)	-	+	-	-
K. pneumoniae (CR2)	-	+	-	+
K. pneumoniae (CR3)	-	-	+	-
Serratia sp. (CB2)	-	+	+	+
B.subtilis (CB3)	-	-	+	+
Klebsiella sp.(CB4)	-	+	+	+
Serratia sp.(OR3)	-	-	+	+
S. saprophyticus (OR5)	-	+	+	+
Klebsiella sp.(OR7)	-	-	-	+
S. marcescens (CD1)	-	+	-	+
Bacillus sp.(CD2)	-	+	+	+
K. pneumoniae (SV1)	-	+	+	-
Endophytic diazotrophs				
K. pneumonia (CGE3)	-	+	-	-
Pantoea sp. (PRE2)	-	+	+	-
Serratia sp. (CRE9)	-	+	+	+
Pseudomonas sp.(CRE10)	-	+	+	+
B.licheniformis (CBE9)	-	-	+	-
Enterobacter sp.(ORE7)	-	+	+	+
P.agglomerans (ORE9)	-	-	+	-
E. sacchari (SVE9)	-	+	+	+
P. fluorescens (Pf1)*	-	+	+	+

+ antagonistic activity; - no antagonistic activity, *standard strain

Table 20. Mineral solubilizing potential of diazotrophic isolates from rhizosphere of grass species collected from different physiographic regions

Isolate	Phosphorus			Potassium			Zinc		
	Colony diameter (mm)	Solubilization zone (mm)	Solubilization efficiency (%)	Colony diameter (mm)	Solubilization zone (mm)	Solubilization efficiency (%)	Colony diameter (mm)	Solubilization zone (mm)	Solubilization efficiency (%)
Enterobacter sp.(BR1)	5.0	12	140 (± 11.64)^{ef}	ND	ND	ND	ND	ND	ND
Klebsiella sp.(CG1)	9.0	12	33 (± 0.98)^{j}	ND	ND	ND	3.0	8.0	167 (± 11.98)^{cd}
Enterobacter sp.(CG3)	4.0	6.0	50 (± 2.17)^{ij}	ND	ND	ND	ND	ND	ND
Bacillus sp. (CG5)	5.0	8.0	60 (± 1.14)^{ij}	ND	ND	ND	ND	ND	ND
Stenotrophomonas sp. (SS4)	4.0	13	225 (± 12.16)^{ab}	ND	ND	ND	ND	ND	ND
K. pneumoniae (CR2)	3.0	9.0	200 (± 2.64)^{bc}	2.0	5.0	150 (± 13.18)^{ab}	4.0	9.0	125 (± 11.64)^{e}
K. pneumoniae (CR3)	4.0	10	150 (± 11.19)^{def}	ND	ND	ND	ND	ND	ND
Serratia sp. (CB2)	4.0	14	250 (± 13.53)^{a}	1.0	3.0	200 (± 13.63)^{a}	4.0	12	200 (± 13.16)^{bc}
B.subtilis (CB3)	4.0	9.0	125 (± 11.29)^{fg}	ND	ND	ND	4.0	13.0	225 (±12.15)^{ab}
Klebsiella sp.(CB4)	4.0	10	150 (± 12.10)^{def}	ND	ND	ND	ND	ND	ND
Serratia sp.(OR3)	3.0	10	233 (±1 3.10)^{a}	3.0	6.0	100 (± 12.17)^{b}	2.0	4.0	100 (± 12.04)^{ef}
S. saprophyticus (OR5)	3.0	8.0	167 (± 11.54)^{de}	ND	ND	ND	ND	ND	ND
Klebsiella sp.(OR7)	3.0	9.0	200 (± 12.64)^{bc}	ND	ND	ND	ND	ND	ND
S. marcescens (CD1)	8.0	14	75(±1.14)^{hi}	2.0	4.0	100 (± 12.14)^{b}	2.0	7.0	250 (± 13.11)^{a}
Bacillus sp.(CD2)	5.0	10	100 (± 13.19)^{gh}	ND	ND	ND	ND	ND	ND
K. pneumoniae (SV1)	3.0	8.0	167 (± 12.63)^{de}	ND	ND	ND	3.0	8.0	167 (± 12.24)^{cd}
P. fluorescens (Pf1*	5.0	15	200 (± 12.69)^{bc}	ND	ND	ND	ND	ND	ND
G. diazotrophicus (PAL5)*	3.0	9.0	200 (± 11.45)^{bc}	ND	ND	ND	4.0	12.0	200 (± 12.11)^{bc}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$) ; standard strain; ND-not detected

Table 21. Mineral solubilizing potential of endophytic diazotrophs isolated from selected grass species collected from different physiographic regions

Isolate	Phosphorus			Potassium			Zinc		
	Colony diameter (mm)	solubilization zone (mm)	Solubilization efficiency (%)	Colony diameter(mm)	solubilization zone (mm)	Solubilization efficiency (%)	Colony diameter (mm)	solubilization zone (mm)	Solubilization efficiency (%)
Pseudomonas sp. (CGE3)	4.0	7.0	75 (± 11.45)^{c}	ND	ND	ND	4.0	12	200 (± 12.74)^{a}
Pantoea sp. (PRE2)	3.0	10	233 (±12.26)^{a}	ND	ND	ND	ND	ND	ND
Serratia sp. (CRE9)	4.0	14	250 (±12.58)^{a}	3.0	6.0	100 (± 11.16)^{b}	3.0	7.0	133 (±12.10)^{cd}
Pseudomonas sp.(CRE10)	4.0	7.0	75 (± 21.45)^{c}	2.0	4.0	100 (± 22.10)^{b}	2.0	4.0	100 (± 12.16)^{d}
B.licheniformis (CBE9)	3.0	8.0	167 (±12.94)^{b}	ND	ND	ND	ND	ND	ND
Enterobacter sp.(ORE7)	3.0	8.0	167 (±12.94)^{b}	ND	ND	ND	ND	ND	ND
P.agglomerans (ORE9)	2.0	6.0	200 (±12.10)^{ab}	3.0	7.0	133 (± 12.14)^{a}	2.0	5.0	150 (± 2.14)^{bc}
E. sacchari (SVE9)	4.0	8.0	100 (± 21.17)^{c}	ND	ND	ND	ND	ND	ND
P. fluorescens (Pf1)*	ND	ND	ND	ND	ND	ND	4.0	11	175 (±12.54)^{ab}
G. diazotrophicus (PAL5)*	3.0	9.0	200 (± 1.45)^{bc}	ND	ND	ND	4.0	12.0	200 (±12.11)^{bc}

ND- solubilization not detected. Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain

Table 22. *In vitro* P- solubilizing potential of diazotrophic isolates from the rhizosphere of grass species collected from different physiographic regions

Isolate	pH	TA (%)	Available P (µg ml^{-1})	Phosphatase (µg of PNP released ml^{-1} day^{-1})
Enterobacter sp.(BR1)	4.0 (± 0.04)^{a}	2.4 (± 0.01)$^{c-g}$	0.47 (± 0.04)^{ghi}	5.8 (± 0.16)^{def}
Klebsiella sp.(CG1)	4.0 (± 0.03)^{a}	3.0 (± 0.03)^{abc}	0.69 (± 0.03)^{cde}	5.3 (± 0.10)^{def}
Enterobacter sp.(CG3)	4.0 (± 0.04)^{a}	2.1 (± 0.05)^{fgh}	0.81 (± 0.01)^{ab}	5.3 (± 0.11)^{def}
Bacillus sp. (CG5)	4.4 (± 0.04)^{a}	1.8 (± 0.01)^{gh}	0.36 (± 0.03)^{hij}	5.3 (± 0.15)^{def}
Stenotrophomonas sp. (SS4)	4.0 (± 0.01)^{a}	1.9 (± 0.01)^{gh}	0.68 (± 0.02)$^{c-f}$	2.4 (± 0.14)^{g}
K. pneumoniae (CR2)	4.0 (± 0.01)^{a}	1.8 (± 0.04)^{gh}	0.56 (± 0.06)^{efg}	4.5 (± 0.11)^{f}
K. pneumoniae (CR3)	4.0 (± 0.00)^{a}	2.9 (± 0.04)$^{a-d}$	0.86 (± 0.01)^{ab}	6.5 (± 0.08)^{cd}
Serratia sp. (CB2)	4.2(± .001)^{a}	2.3 (± 0.03)$^{d-g}$	0.68 (± 0.04)$^{c-f}$	5.4 (± 0.34)^{def}
B.subtilis (CB3)	4.3 (± 0.01)^{a}	3.1 (± 0.14)^{ab}	0.54 (± 0.01)^{efg}	4.4 (± 0.21)^{f}
Klebsiella sp.(CB4)	4.0 (± 0.04)^{a}	2.2 (±0.05)$^{e-h}$	0.25 (± 0.01)^{j}	5.6 (± 0.26)^{def}
Serratia sp.(OR3)	4.0 (± 0.04)^{a}	3.4 (± 0.01)^{a}	0.26 (± 0.01)^{j}	5.3 (± 0.25)^{def}
S. saprophyticus (OR5)	4.0 (± 0.06)^{a}	2.9 (± 0.04) $^{a-d}$	0.35 (± 0.03)^{ij}	4.5 (± 0.04)^{f}
Klebsiella sp.(OR7)	4.0 (± 0.14)^{a}	3.2 (± 0.02)^{ab}	0.36 (± 0.02)^{hij}	4.4 (±0.16)^{f}
S. marcescens (CD1)	4.0 (± 0.01)^{a}	2.3 (± 0.05)$^{d-g}$	0.85 (± 0.04)^{ab}	12.9 (± 0.10)^{a}
Bacillus sp.(CD2)	4.0 (± 0.05)^{a}	3.1 (± 0.06)^{ab}	0.96 (± 0.09)^{a}	10.9 (± 0.29)^{b}
K. pneumoniae (SV1)	4.0 (± 0.07)^{a}	2.8 (± 0.08)$^{a-e}$	0.48 (± 0.03)^{ghi}	7.5 (± 0.24)^{c}
P. fluorescens (Pf1)*	4.0 (± 0.03)^{a}	2.3 (± 0.02)$^{d-g}$	0.75 (± 0.01)^{bcd}	5.6 (± 0.08)^{def}
Control	6.6 (± 0.01)^{b}	0.2 (± 0.06)^{i}	0.02 (± 0.04)^{k}	0.05 (± 0.02)^{h}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain

Table 23. *In vitro* P- solubilizing potential of endophytic diazotrophic isolates of grass species collected from different physiographic regions

Isolate	pH	TA (%)	Available P (µg ml^{-1})	Phosphatase (µg of PNP ml^{-1} day^{-1})
Pseudomonas sp. (CGE3)	4.1 (± 0.02)^{a}	2.6 (± 0.01)^{bcd}	0.56 (± 0.02)^{c}	8.0 (± 0.07)^{ab}
Pantoea sp. (PRE2)	4.4 (± 0.02)^{a}	2.4(± 0.04)^{bcd}	0.36 (± 0.04)^{de}	7.3 (± 0.09)^{b}
Serratia sp. (CRE9)	4.0(±0.01)^{a}	2.5 (± 0.08)^{bcd}	0.56 (± 0.03)^{c}	6.4 (± 0.15)^{bc}
Pseudomonas sp.(CRE10)	4.2 (± 0.04)^{a}	2.9 (± 0.01)^{a}	0.78 (± 0.01)^{a}	7.6 (± 0.11)^{b}
Bacillus sp.(CBE9)	4.3 (± 0.05)^{a}	2.4 (± 0.08)^{bcd}	0.40(±0.02)^{d}	3.3 (± 0.11)^{e}
Enterobacter sp.(ORE7)	4.2 (± 0.04)^{a}	2.4 (± 0.09)^{bcd}	0.65(± 0.05)^{bc}	5.5 (± 0.04)^{cd}
P.agglomerans (ORE9)	4.2 (± 0.02)^{a}	2.6 (± 0.04)^{bcd}	0.56 (±0.04)^{c}	8.6 (± 0.11)^{a}
E. sacchari (SVE9)	4.3 (± 0.05)^{a}	2.7 (±0.02)^{bc}	0.23 (± 0.01)^{e}	2.8 (± 0.10)^{e}
P. fluorescens (pf1) *	4.6 (± 0.01)^{a}	2.1 (± 0.08)^{cd}	0.58 (± 0.03)^{c}	8.1 (± 0.16)^{ab}
Control	6.6 (± 0.01)^{b}	0.2 (± 0.06)^{e}	0.02 (± 0.04)^{f}	0.05 (± 0.02)^{f}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain

Among the ten endophytic isolates, *Serratia* sp. (CRE9) caused maximum (4.0 ± 0.01) pH reduction. The titrable acidity was found to be increased due to the growth of phosphorus solubilizing organisms. Maximum titrable acidity of 2.9 ±0.01 per cent was present in the culture filtrate of *Pseudomonas* sp. (CRE10) (Table. 23) after nine days of incubation. The amount of available P was high in *Pseudomonas* sp. (CRE10) which produced 0.78 ± 0.01 µg ml^{-1}. The phosphatase activity was the highest in the isolate P. *agglomerans* (ORE9) (8.6±0.11 µg of PNP released ml^{-1} day^{-1}).

4.10.2. *In vitro* potassium solubilizing potential of diazotrophic isolates

The ability of rhizosphere and endophytic diazotrophs to solubilize potassium was determined by observing solubilization zone in Alexandrov medium. Only 4 rhizosphere diazotrophs were able to solubilize potassium. Maximum solubilzation efficiency was recorded in *Serratia* sp. (CB2) (200 ± 13.63 percent) (Table 20). Among the endophytes, three isolates showed positive result for K solubilization. Highest solubilzation efficiency was observed in *P. agglomerans* (ORE9) (133 ± 12.14 percent) (Table 21).

Influence of potassium solubilizing organisms on pH, titrable acidity and available potassium growth medium are mentioned in Table 24. In general, the pH of the growth medium was decreased by the growth of all potassium solubilizing isolates. Maximum titrable acidity of 2.9 ± 0.02 per cent and 2.7±0.01 was present in the culture filtrate of *Serratia* sp. (CR2) and *Serratia* sp.(OR3) respectively. The available potassium was found to be highest in *K. pneumoniae* (CR2) (0.35±0.03µg ml^{-1}) (Table 24).

Similarly, pH of the growth medium was decreased by the growth of all potassium solubilizing endophytic isolates and the decline in the pH increased with incubation period. Among the isolates, *Serratia* sp. (CRE9) (4.0 ± 0.04) caused maximum decline in the pH. Maximum titrable acidity of 2.5 ± 0.03 and 2.4 ± 0.02 per cent was present in the culture filtrate of *P. agglomerans* (ORE9) and *Pseudomonas* sp. (CRE10) respectively. The isolate *P. agglomerans* (ORE9) registered maximum amount of the available K (0.14±0.04 µg ml^{-1}) (Table. 24).

Table 24. *In vitro* potassium releasing potential of diazotrophic isolates from grass species

Isolate	pH	TA (%)	Available K (µg ml^{-1})
Rhizosphere diazotrophs			
S. marcescens (CD1)	4.3 (± 0.01)^{a}	2.4 (± 0.04)^{b}	0.12 (± 0.01)^{c}
Serratia sp. (CB2)	4.4 (± 0.02)^{a}	2.0 (± 0.05)^{b}	0.13 (± 0.02)^{b}
K. pneumonia (CR2)	4.2 (± 0.01)^{a}	2.9 (± 0.02)^{a}	0.35 (± 0.03)^{a}
Serratia sp.(OR3)	4.3 (± 0.01)^{a}	2.7 (± 0.01)^{a}	0.12 (± 0.01)^{bc}
Endophytic diazotrophs			
P.agglomerans (ORE9)	4.1 (± 0.04)^{a}	2.4 (± 0.02)^{a}	0.14 (± 0.04)^{a}
Serratia sp. (CRE9)	4.0 (± 0.04)^{a}	2.1 (± 0.05)^{ab}	0.12 (± 0.04)^{b}
Pseudomonas sp.(CRE10)	4.3 (± 0.01)^{a}	2.5 (± 0.03)^{a}	0.13 (± 0.03)^{b}
Control	5.4 (± 0.01)^{b}	0.7 (± 0.02)^{c}	0.04 (± 0.01)^{c}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$).

4.10.3. *In vitro* zinc solubilizing potential of diazotrophic isolates

It was observed that 7 isolates out of total 16 were able to solubilize zinc oxide. The highest solubilization efficiency was found with *Klebsiella* sp. (OR5) (250 ± 13.11%) (Table 20). Among the endophytes, *Pseudomonas* sp. (CGE3) showed maximum solubilizing efficiency of 200 ± 2.74 per cent (Table 21).

Influence of Zinc solubilizing organism on pH, titrable acidity and available zinc of the medium are presented in table 25. The isolate *Serratia* sp. (OR3) caused maximum decline in the pH (3.8 ±0.04) while, the titrable acidity of the medium showed an increase due to the growth of zinc solubilizing organisms. Maximum titrable acidity of (2.8 ± 0.08) per cent was present in the culture filtrate of *Staphylococcus saprophyticus*. (OR5) and the maximum available zinc was observed in *Staphylococcus saprophyticus* (OR5) (0.60 ±0.04 µg ml^{-1}) (Table. 25).

In endophytes, the maximum decline in pH (4.0 ± 0.04), increase in titrable acidity (2.8±0.04 per cent) and maximum amount of available zinc was found in *Serratia* sp. (CRE9) (0.60 ± 0.04 µg ml^{-1}) (Table 25).

Table 25. *In vitro* zinc solubilizing potential of diazotrophic isolates from grass species

Isolate	pH	TA (%)	Available Zinc (µg ml^{-1})
Rhizosphere diazotrophs			
Enterobacter sp.(BR1)	4.6 (± 0.04)^{c}	2.1 (± 0.03)$^{b-e}$	0.22 (± 0.04)^{ef}
K. pneumoniae (CR2)	4.8 (± 0.08)^{c}	1.9 (± 0.01)^{de}	0.32 (± 0.03)^{d}
Klebsiella sp. (CB4)	4.6 (± 0.02)^{c}	1.7 (± 0.05)^{e}	0.20 (± 0.06)$^{e-h}$
Serratia sp. (OR3)	3.8 (± 0.04)^{a}	2.4 (± 0.10)$^{a-d}$	0.56 (± 0.06)^{b}
S. saprophyticus.(OR5)	4.9 (± 0.04)^{c}	2.8 (± 0.08)^{a}	0.60 (± 0.04)^{a}
S. marcescens (CD1)	4.2 (± 0.06)^{b}	2.0 (± 0.06)^{cde}	0.22 (± 0.06)^{ef}
Bacillus sp.(CD2)	4.3 (± 0.04)^{b}	2.1 (± 0.04)$^{b-e}$	0.21 (± 0.05)^{efg}
Endophytic diazotrophs			
Pseudomonas sp.(CGE3)	5.2 (± 0.04)^{b}	2.0 (± 0.05)^{b}	0.12 (± 0.04)^{c}
P. agglomerans (ORE9)	5.1 (± 0.01)^{c}	2.3 (± 0.03)^{ab}	0.22 (± 0.01)^{c}
Serratia sp. (CRE9)	4.0 (± 0.04)^{a}	2.8 (± 0.04)^{a}	0.60 (± 0.04)^{a}
Pseudomonas sp.(CRE10)	4.0 (± 0.04)^{a}	2.7 (± 0.04)^{a}	0.54 (± 0.04)^{a}
G.diazotrophicus (PAL5)*	4.3 (± 0.07)^{ab}	2.7 (± 0.05)^{a}	0.43 (± 0.04)^{b}
Control	5.1 (± 0.04)^{b}	0.6 (± 0.01)^{c}	0.03 (± 0.02)^{d}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain

4.11. Stress tolerance by diazotrophic isolates from selected grass species

4.11.1. Salt stress on growth of diazotrophic isolates

The salt tolerance ability of the diazotrophic isolates were tested by their growth at different salt concentrations *viz.*, 1%, 2.5%, 5.0% and 7.5%.From the results it was observed that, all the 16 isolates were promising for their ability to tolerate upto 5%, especially three isolates *viz*., *Bacillus* sp. (CG5), *Stenotrophomonas* sp. (SS4) and *Bacillus* sp. (CD2) registered their remarkable ability to tolerate upto 7.5%. (Table 26). Among the endophytes, 3 isolates namely *E. sacchari* (SVE9), *Bacillus* sp. (CBE9) and *Serratia* sp. (CRE9) were able to tolerate salt concentration of 7.5% while the remaining were able to tolerate up to 5% (Table 27).

From the results it was observed that the diazotrophic population significantly influenced by various salts concentrations. The diazotrophic population load at different salt concentration with different time interval (0, 12, 24, 36 and 46 h) are given in annexure I.

4.11.2. Temperature stress on growth of diazotrophic isolates

The temperature had profound influence on the population of diazotrophs. It was observed that the five cultures *Bacillus* sp (CG5), *Stenotrophomonas* sp. (SS4), *K. pneumoniae* (CR3), *Bacillus* sp.(CD2) and *B. subtilis* (CB3) were highly temperature tolerant with growth even at 55^{o}C (Table 28). Among the endophytes three isolates namely, *Serratia* sp. (CRE9), *Enterobacter* sp. (ORE7) and *E. sacchari* (SVE9) were able to tolerate upto 55^{o}C. (Table 29). From the results it was observed that the diazotrophic population significantly influenced by various temperature levels. The diazotrophic population load at different temperature with different time interval (0, 12, 24, 36 and 46 h) are given in annexure I.

4.11.3. Soil moisture potential on survival of diazotrophic isolates

From the results, it was observed that *Enterobacter* sp. (CG3) recorded the maximum population (7.23 ± 0.36) at moisture potential of 1 BAR (field capacity). Among endophytes *Pseudomonas* sp. (CGE3) produced the higher population (7.62 ± 0.46 log cfu ml^{-1}) at moisture potential of 15 BAR (permanent wilting point). However these two moisture

Table 26. Effect of salt (NaCl) concentrations on the growth of diazotrophic isolates from the rhizosphere of grass species

Isolate	Population (log cfu ml^{-1}) at different levels salt concentration			
	1% NaCl	2.5% NaCl	5.0% NaCl	7.5% NaCl
Enterobacter sp.(BR1)	6.79 (± 0.28)^{a}	5.12 (± 0.49)^{n}	3.98 (± 0.48)^{u}	ND
Klebsiella sp.(CG1)	6.45 (± 0.45)^{b}	5.82 (± 0.29)^{ijk}	4.31 (± 0.34)^{s}	ND
Enterobacter sp.(CG3)	6.17 (± 0.56)^{efg}	6.04 (± 0.34)^{gh}	4.88 (± 0.32)^{op}	ND
Bacillus sp. (CG5)	5.78 (± 0.88)^{jk}	4.72 (±0.42)^{qr}	3.95 (± 0.39)^{u}	3.60 (± 0.17)^{v}
Stenotrophomonas sp.(SS4)	6.14 (± 0.78)^{efg}	5.67 (± 0.45)^{kl}	4.57 (± 0.56)^{r}	2.42 (± 0.45)^{w}
K. pneumoniae (CR2)	5.91 (± 0.88)^{hij}	5.03 (±0.56)^{no}	4.97 (± 0.35)^{no}	ND
K. pneumoniae (CR3)	6.25 (± 0.34)^{de}	4.78 (± 0.34)^{pq}	5.06 (± 0.56)^{n}	ND
Serratia sp. (CB2)	6.23 (± 0.67)^{def}	5.48 (± 0.98)^{m}	4.67 (± 0.43)^{qr}	ND
B.subtilis (CB3)	6.42 (± 0.23)^{bc}	5.52 (± 0.56)^{lm}	3.72 (± 0.46)^{v}	ND
Klebsiella sp.(CB4)	6.37 (± 0.68)^{bcd}	4.70 (± 0.78)^{qr}	3.82 (± 0.86)^{uv}	ND
Serratia sp.(OR3)	6.16 (± 0.20)^{efg}	5.46 (± 0.58)^{m}	4.14(± 0.58)^{t}	ND
S. saprophyticus (OR5)	6.74 (± 0.37)^{a}	5.45 (± 0.49)^{m}	3.67 (± 0.38)^{v}	ND
Klebsiella sp.(OR7)	6.08 (± 0.67)^{fgh}	5.44 (± 0.23)^{m}	3.98 (± 0.56)^{u}	ND
S. marcescens (CD1)	6.22 (± 0.60)^{def}	6.28 (± 0.56)^{cde}	3.95 (± 0.45)^{u}	ND
Bacillus sp.(CD2)	5.95 (± 0.63)^{hi}	4.98 (± 0.24)^{no}	3.76 (± 0.27)^{v}	2.68 (± 0.22)^{w}
K. pneumoniae (SV1)	6.30 (± 0.29)^{bcde}	5.73 (± 0.34)^{k}	4.22 (± 0.45)st	ND
*A.lipoferum (*Az 204*)**	6.03 (± 0.56)^{gh}	5.51 (± 0.48)^{lm}	0.00 (± 0.56)^{z}	ND
	SEd		CD ($p \leq 0.05$)	
Isolates	0.23		0.47**	
Conc.	0.21		0.42**	
I X C	0.47		0.93**	

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); * standard strain

Table 27. Effect of salt (NaCl) concentration on the growth of endophytic diazotrophic isolates from grass species

Isolate	Population (log cfu ml^{-1}) at different level of salt concentration (NaCl %)			
	1% NaCl	2.5% NaCl	5.0% NaCl	7.5% NaCl
Pseudomonas sp. (CGE3)	6.35 (± 0.58)^{a}	5.64 (± 0.34)^{cde}	4.03 (± 0.67)^{qt}	ND
Pantoea sp. (PRE2)	5.59 (± 0.67)^{def}	5.79 (± 0.29)^{bc}	4.73 (± 0.85)^{l}	ND
Serratia sp. (CRE9)	5.47 (± 0.23)^{fgh}	5.37 (± 0.78)^{ghi}	4.98 (± 0.47)^{k}	3.79 (± 0.22)^{s}
Pseudomonas sp.(CRE10)	5.31 (± 0.25)^{hij}	4.52 (± 0.45)^{mn}	4.41 (± 0.45)^{mno}	ND
Bacillus sp.(CBE9)	4.99 (± 0.45)^{k}	4.56 (± 0.23)^{m}	4.37 (± 0.37)^{no}	2.64 (± 0.45)^{u}
Enterobacter sp.(ORE7)	5.71 (± 0.24)^{bcd}	4.43 (± 0.54)^{mno}	5.44 (± 0.22)^{fgh}	ND
P.agglomerans (ORE9)	5.71 (± 0.22)^{bcd}	5.26 (± 0.34)^{ij}	4.14 (± 0.21)^{pq}	ND
E. sacchari (SVE9)	5.74 (± 0.45)^{bcd}	4.53 (± 0.67)^{mn}	3.94 (± 0.37)^{rs}	2.85 (± 0.24)^{t}
G. diazotropicus (PAL5)*	5.53 (± 0.56)^{efg}	5.16 (± 0.56)^{j}	4.27 (± 0.22)^{op}	ND
	SEd		CD ($p \leq 0.05$)	
Isolates	0.25		0.51*	
Conc.	0.23		0.45**	
I X C	0.51		1.01**	

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); * standard strain

Table 28. Effect of temperature on the growth of diazotrophs isolated from the rhizosphere of selected grass species

Isolate	Population (log cfu ml^{-1}) at different temperature levels			
	40 ºC	45 ºC	50 ºC	55 ºC
Enterobacter sp.(BR1)	6.53 (± 0.34)^{fg}	5.10 (± 0.45)^{p}	5.47 (± 0.34)^{n}	ND
Klebsiella sp.(CG1)	6.87 (± 0.54)^{b}	5.58 (± 0.38)^{lmn}	3.95 (± 0.47)^{v}	ND
Enterobacter sp.(CG3)	6.26 (± 0.34)^{hi}	6.14 (± 0.54)^{ij}	4.29 (± 0.67)^{t}	ND
Bacillus sp. (CG5)	6.15 (± 0.45)^{ij}	6.01(± 0.23)^{jk}	4.86 (± 0.56)^{qr}	3.59 (± 0.34)^{w}
Stenotrophomonas sp. (SS4)	6.51 (± 0.34)^{fg}	4.89 (± 0.34)^{qr}	4.15 (± 0.48)^{tu}	4.29 (± 0.51)^{t}
K. pneumoniae (CR2)	5.94 (± 0.88)^{k}	5.63 (± 0.56)^{lmn}	4.91 (± 0.54)^{qr}	ND
K. pneumoniae (CR3)	6.70 (± 0.38)^{de}	5.01 (± 0.67)^{pq}	4.95 (± 0.18)^{qr}	2.38 (± 0.52)^{y}
Serratia sp. (CB2)	6.89 (± 0.57)^{a}	4.89 (± 0.45)^{qr}	4.81 (± 0.26)^{r}	ND
B.subtilis (CB3)	6.63 (± 0.52)^{ef}	5.67 (± 0.51)^{lm}	4.61 (± 0.58)^{s}	2.67 (± 0.23)^{x}
Klebsiella sp.(CB4)	6.30 (± 0.50)^{hi}	5.50 (± 0.53)^{mn}	4.04 (± 0.52)^{uv}	ND
Serratia sp.(OR3)	6.29 (± 0.23)^{hi}	5.29 (± 0.45)^{o}	3.61 (± 0.45)^{w}	ND
S. saprophyticus (OR5)	6.63 (± 0.35)^{ef}	5.47 (± 0.34)^{n}	4.01(± 0.98)^{uw}	2.67 (± 0.21)^{x}
Klebsiella sp.(OR7)	6.51 (± 0.45)^{fg}	5.70 (± 0.67)^{i}	3.64 (± 0.51)^{w}	ND
S. marcescens (CD1)	6.83 (± 0.37)^{cd}	6.24 (± 0.46)^{hi}	3.93 (± 0.34)^{v}	ND
Bacillus sp.(CD2)	5.94 (± 0.32)^{k}	6.50 (± 0.16)^{fg}	3.88 (± 0.45)^{v}	ND
K. pneumoniae (SV1)	6.51 (± 0.28)^{fg}	5.13 (± 0.52)^{p}	3.71 (± 0.53)^{w}	ND
A.lipoferum (Az 204)*	6.37 (± 0.17)^{gh}	7.10 (±0.23)^{b}	9.10 (± 0.34)^{a}	ND
	SEd	CD ($p \leq 0.05$)		
Isolates	0.25	0.49**		
Conc.	0.22	0.44**		
I X C	0.49	0.98**		

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); *standard strain

Table 29. Effect of temperature on the growth of endophytic diazotrophs isolated from the selected grass species

Isolate	Population (log cfu ml^{-1}) at different level of temperature			
	40 ºC	45 ºC	50 ºC	55 ºC
Pseudomonas sp. (CGE3)	6.26 (± 0.34)^{a}	5.60 (± 0.22)^{de}	4.28 (± 0.21)^{o}	ND
Pantoea sp. (PRE2)	5.48 (± 0.23)^{efg}	5.56 (± 0.45)^{def}	5.00 (± 0.25)^{kl}	ND
Serratia sp. (CRE9)	5.43 (± 0.43)^{fgh}	5.36 (± 0.45)^{gh}	4.95 (± 0.48)^{lm}	4.04 (± 0.54)^{p}
Pseudomonas sp.(CRE10)	6.18 (± 0.56)^{a}	4.53 (± 0.56)^{n}	4.41 (± 0.78)^{no}	ND
Bacillus sp.(CBE9)	5.71 (± 0.46)^{cd}	4.43 (± 0.23)^{no}	5.59 (± 0.46)^{def}	3.01 (± 0.48)^{r}
Enterobacter sp.(ORE7)	5.55 (± 0.43)^{def}	4.53 (± 0.34)^{n}	4.37 (± 0.24)^{no}	ND
P.agglomerans (ORE9)	5.83 (± 0.43)^{bc}	5.11 (± 0.45)^{jk}	4.32 (± 0.45)^{o}	ND
E. sacchari (SVE9)	5.48 (± 0.68)^{efg}	4.43 (± 0.34)^{no}	4.05 (± 0.34)^{p}	3.25 (± 0.37)^{q}
G. diazotropicus (PAL5)*	6.28 (± 0.89)^{a}	5.16 (± 0.34)^{ij}	3.22 (± 0.23)^{q}	ND
	SEd		CD ($p \leq 0.05$)	
Isolates	0.25		0.49*	
Conc.	0.22		0.44**	
I X C	0.50		0.98**	

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); * standard strain

Table 30. Effect of different soil moisture potentials on the growth of diazotrophic isolates from rhizosphere of grass species

Isolate	Population (log cfu ml^{-1}) at different level of moisture potential	
	Field capacity (1 BAR)	Permanent wilting point (15 BAR)
Enterobacter sp.(BR1)	6.77 (± 0.34)^{def}	6.56 (± 0.31)^{ghij}
Klebsiella sp.(CG1)	6.93 (± 0.42)^{bcd}	7.00 (± 0.36)^{bc}
Enterobacter sp.(CG3)	7.23 (± 0.36)^{a}	7.09 (± 0.58)^{ab}
Bacillus sp. (CG5)	6.85 (± 0.56)^{cde}	6.09 (± 0.42)^{pq}
Stenotrophomonas sp. (SS4)	6.72 (± 0.45)^{efg}	6.54 (± 0.36)^{hijk}
K. pneumoniae (CR2)	6.67 (± 0.43)^{fgh}	6.01 (± 0.47)^{q}
K. pneumoniae (CR3)	6.99 (± 0.34)^{bc}	6.57 (± 0.57)^{ghij}
Serratia sp. (CB2)	6.98 (± 0.23)^{bc}	6.89 (± 0.79)^{cde}
B.subtilis (CB3)	6.75 (± 0.21)^{ef}	6.62 (± 0.57)^{fghi}
Klebsiella sp.(CB4)	6.27 (± 0.47)^{mno}	6.44 (± 0.39)^{jkl}
Serratia sp.(OR3)	6.32 (± 0.45)^{lmn}	6.23 (± 0.31)^{mnop}
S. saprophyticus (OR5)	6.44 (± 0.78)^{jkl}	6.63(± 0.28)^{fghi}
Klebsiella sp.(OR7)	6.45 (± 0.46)^{jkl}	6.87 (± 0.45)^{cde}
S. marcescens (CD1)	6.44 (± 0.34)^{jkl}	6.77 (± 0.46)^{def}
Bacillus sp.(CD2)	6.49 (± 0.56)^{ijk}	6.11 (± 0.68)^{opq}
K. pneumoniae (SV1)	6.39 (± 0.46)^{klm}	6.58 (± 0.54)^{ghij}
A.lipoferum (Az 204) *	6.24 (± 0.33)^{mnop}	6.20 (± 0.43)^{nop}
	SEd	CD ($p \leq 0.05$)
Isolates	0.42	0.83
Conc.	0.26	0.52
I X C	0.59	1.16

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); * standard strain

Table 31. Effect of soil moisture potential on the growth of endophytic diazotrophs isolated from selected grass species

Isolate	Population (log cfu ml^{-1}) at different level of moisture potential	
	Field capacity (1 BAR)	Permanent wilting point (15 BAR)
Pseudomonas sp. (CGE3)	7.34 (± 0.34)^{b}	7.62 (± 0.46)^{a}
Pantoea sp. (PRE2)	6.24 (± 0.45)^{ij}	6.61 (± 0.57)^{fgh}
Serratia sp. (CRE9)	6.13 (± 0.34)^{ijk}	6.48 (± 0.64)^{h}
Pseudomonas sp.(CRE10)	6.91 (± 0.26)^{d}	7.10 (± 0.45)^{c}
Bacillus sp. (CBE9)	6.21 (± 0.43)^{ij}	6.70 (± 0.78)^{ef}
Enterobacter sp. (ORE7)	6.27 (± 0.56)^{i}	6.48 (± 0.89)^{h}
P. agglomerans (ORE9)	6.51 (± 0.65)^{gh}	6.47 (± 0.47)^{h}
E. sacchari (SVE9)	5.99 (± 0.56)^{kl}	6.09 (± 0.58)^{jk}
G. diazotropicus (PAL5) *	5.88 (± 0.46)^{l}	6.11 (± 0.59)^{ijk}
	SEd	CD ($p \leq 0.05$)
Isolates	0.37	0.73
Conc.	0.23	0.46
I X C	0.51	1.03

Values are mean (± SE) (n=3) and values followed by the same letter are not significantly different from each other as detected by DMRT ($p \leq 0.05$); * standard strain

potential were not found to significantly influence the population of diazotrophs (Table 30 and 31). The diazotrophic population load at different level of moisture potential in 1, 15, 30, 45, 60 days interval are given in Annexure I.

4.11.4. Heavy metal tolerance by diazotrophic isolates

The maximum tolerable concentration (MTC) of 5 different heavy metal ions *viz.*, Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} by diazotrophic isolates was estimated by streaking the cultures on to LB medium containing heavy metal ions. The isolates were found to have very low resistance to Cd^{2+} and Hg^{2+} than the other metals. Among the 16 isolates, 9 were able to exhibit the resistance to Zn^{2+} while 8 were resistance against Co^{2+}, Ni^{2+} (Table 32).

From the results, it was observed that endophytes were less resistant to Ni^{2+} and more resistant were observed against Cd^{2+} Hg^{2+}, Co^{2+} and Zn^{2+} (Table 32).

4.12. Effect of growth promoting diazotrophs inoculation on the growth parameters of rice seedlings (cultivar- ADT 43) under *in vitro* condition

In order to evaluate the performance of 15 multiple growth promoting diazotrophic isolates (Table 33), rice seedlings were raised under gnotobiotic conditions along with commercial strain (*A. lipoferum* - Az 204) as standard (Plate 6).

4.12.1. Effect of on biometric characteristics of rice seedlings

Among the isolates the maximum shoot length was observed in *Pseudomonas* sp. (CRE10) (19.5 ±1.21cm) inoculated treatment. The uninoculated control recorded the lower shoot length (11.9 ±1.22 cm) while, higher root length was recorded in *Pseudomonas* sp. (CRE10) and *K. pneumonia* (CR2) that were onpar with 9.6 cm. The least root length was observed in control (5.8±0.59 cm). Plant dry matter production was higher in *S. marcescens* (CD1) (50.0 ±0.02mg). The growth attributes of rice seedlings are presented in Fig. 9.

4.12.2. Localization and distribution of endophytic bacteria on roots and culms of rice seedlings

The endophytic colonization in the rice seedlings (roots and culm) were examined by SEM analysis. Results indicated that endophytic bacteria were preferentially colonized in the rhizoplane of rice roots (Plate7a-d). Diazotrophic cells were observed in the longitudinal and horizontal sectioned roots and culms.

Table 32. Maximum tolerable concentration (MTC) of heavy metals by diazotrophic isolates from grass species

Isolates	Heavy metal (μg ml^{-1})				
	Cd^{2+}	Hg^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}
Rhizosphere diazotrophs					
Enterobacter sp.(BR1)	ND	ND	G_{600}	ND	G_{100}
Klebsiella sp.(CG1)	ND	ND	G_{100}	G_{100}	G_{100}
Enterobacter sp.(CG3)	ND	100	ND	G_{100}	G_{200}
Bacillus sp. (CG5)	G_{100}	G_{100}	G_{100}	G_{200}	ND
Stenotrophomonas sp. (SS4)	ND	ND	G_{100}	G_{100}	G_{100}
K. pneumoniae (CR2)	ND	G_{100}	ND	ND	ND
K. pneumoniae (CR3)	ND	G_{200}	G_{800}	G_{100}	ND
Serratia sp. (CB2)	ND	ND	ND	ND	ND
B.subtilis (CB3)	G_{100}	ND	G_{100}	ND	G_{100}
Klebsiella sp.(CB4)	ND	ND	ND	ND	G_{100}
Serratia sp.(OR3)	ND	ND	ND	G_{100}	G_{100}
S. saprophyticus (OR5)	ND	ND	G_{400}	G_{100}	ND
Klebsiella sp.(OR7)	ND	ND	ND	G_{200}	G_{200}
S. marcescens (CD1)	ND	ND	ND	ND	ND
Bacillus sp.(CD2)	G_{600}	ND	G_{100}	G_{100}	ND
K. pneumoniae (SV1)	G_{200}	G_{400}	G_{100}	ND	ND
A.lipoferum (Az 204) *	ND	G_{100}	G_{200}	ND	G_{400}
Endophytic diazotrophs					
Pseudomonas sp. (CGE3)	ND	ND	ND	G_{100}	ND
Pantoea sp. (PRE2)	G_{100}	ND	ND	G_{200}	ND
Serratia sp. (CRE9)	G_{100}	ND	G_{200}	G_{100}	ND
Pseudomonas sp.(CRE10)	ND	G_{100}	G_{200}	G_{100}	ND
Bacillus sp.(CBE9)	ND	G_{100}	G_{100}	G_{200}	G_{200}
Enterobacter sp.(ORE7)	G_{200}	ND	ND	ND	ND
P.agglomerans (ORE9)	G_{100}	G_{100}	G_{400}	ND	ND
E. sacchari (SVE9)	G_{400}	G_{200}	G_{100}	ND	G_{200}
G.diazotropicus (PAL5) *	G_{100}	G_{100}	ND	ND	ND

The subscript numeral succeding the alphabet G indicate the maximum tolerable concentration of heavy metal by the isolate ; *standard strain; ND- not detected

Table 33. Multifaceted plant growth promoting activities of diazotrophic isolates used for gnotobiotic study in rice

Isolate	N-fixation		Plant growth promoting activity						Mineral solubilisation			Stress tolerance			
	ARA	*nifH* gene	IAA	GA	ACCD	Siderophore	HCN	[a]ANT	P	K	Zn	Temperature (>45º C)	Salt (>2.5%)	Drought (15BAR)	[b]Heavy metal
Klebsiella sp.(CG1)	+	+	+	+	ND	+	+	$+^2$	+	ND	+	+	+	+	$+^3$
Enterobacter sp.(CG3)	+	+	+	+	ND	+	+	+	+	ND	ND	+	+	+	$+^3$
Bacillus sp (CG5)	+	+	+	+	ND	+	+	+	+	ND	ND	+	+	+	$+^3$
S. marcescens (CD1)	+	+	+	+	+	+	ND	$+^2$	+	+	+	+	+	+	$+^4$
Serratia sp. (CB2)	+	+	+	+	+	+	+	$+^3$	+	+	+	+	+	+	$+^2$
B.subtilis (CB3)	+	+	+	+	ND	+	ND	$+^2$	+	ND	+	+	+	+	+
K. pneumoniae (CR2)	+	+	+	+	+	+	+	$+^2$	+	+	+	+	+	+	+
K. pneumoniae (CR3)	+	+	+	+	ND	+	+	+	+	ND	ND	+	+	+	$+^2$
K. pneumoniae (SV1)	+	+	+	+	+	+	ND	$+^2$	+	ND	+	+	+	+	$+^2$
Serratia sp.(OR3)	+	+	+	+	+	+	+	$+^2$	+	+	+	+	+	+	$+^3$
Serratia sp. (CRE9)	+	+	+	+	ND	+	+	$+^3$	+	+	+	+	+	+	$+^3$
Pseudomonas sp.(CRE10)	+	+	+	+	+	+	+	$+^3$	+	+	+	+	+	+	$+^4$
Pseudomonas sp.(CGE3)	+	+	+	+	ND	+	ND	+	+	ND	+	+	+	+	+
P.agglomerans (ORE9)	+	+	+	+	+	+	+	$+^3$	+	+	+	+	+	+	$+^3$
A.lipoferum (Az 204) *	+	+	+		ND	+	+	ND	ND	ND	ND	ND	+	+	+
P.fluorescens (Pf1) *	+	ND	+	+	+	+	+	$+^3$	+	ND	ND	ND	ND	ND	ND
G.diazotrophicus (PAL5) *	+	ND	ND	ND	ND	ND	ND	ND	+	ND	+	ND	+	+	$+^2$

*standard strain,[a] The + sign followed by number in superscript indicate the number of plant pathogens to which the isolate shows antagonistic activity ;
[b] The + sign followed by number indicate the number of heavy metals to which the isolate is tolerant; ND- not detected; ANT- Antaganism

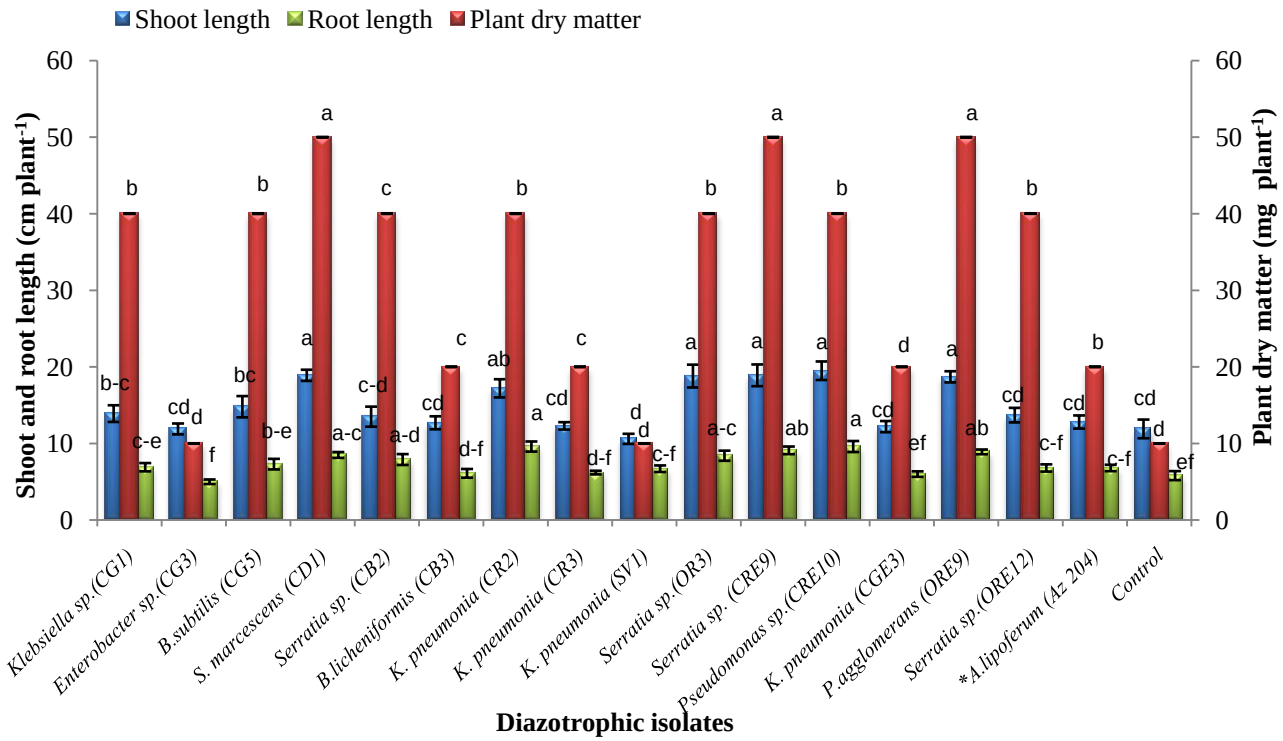

Fig 9. Effect of inoculation diazotrophic isolates on growth of rice seedlings. Data are presented as mean ± SE (n=3); For each panel, different letters indicate significantly different at P≤0.05 according to Duncan's Multiple Range Test.

Plate 6. Gnotobiotic experiment to study the effect of inoculation of diazotrophic isolates on the growth of rice (cultivar-ADT 43)

The diazotrophic isolates *Klebsiella* sp.(CG1) *Enterobacter* sp.(CG3), *Bacillus* sp. (CG5), *S. marcescens* (CD1), *Serratia* sp. (CB2), *B. subtilis* (CB3), *K. pneumonia* (CR2), *K. pneumonia* (CR3), *K. pneumonia* (SV1), *Serratia* sp.(OR3), *Serratia* sp. (CRE9), *Pseudomonas* sp.(CRE10), *Pseudomonas* sp.(CGE3) and *P. agglomerans* (ORE9) showed the better performance than the standard strain (Az 204) in gnotobiotic conditions. On the basis of *in vitro* performance the above isolates were taken for field study.

4.12. Crop response study to evaluate the efficiency of elite bacteria under *in vivo* conditions

A field experiment was carried out at the Wetland Farm, Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore to evaluate the efficiency of elite bacteria on the growth and yield of rice (cultivar- ADT 43) under lowland ecosystem (Plate 8). The results of the experiments are elucidated in the following sections.

4.12.1. Effect of diazotrophic isolates on growth of rice

The plant height (cm) increased with the advancement of crop growth from tillering stage and it reached the maximum at harvest but after flowering stage the rate of increase was rather negligible. From the results, taller plants were observed in T4 –with *Serratia* sp. (CB2) (58.54±2.27 cm). However, the treatments T1, T8, T9 and T10 were on par. Similar trend were maintained at panicle imitation stage. At maturity stage higher plant height was observed in T1 and T8. The treatment T12 (absolute control) produced shorter plants at all the growth stages of observation (Table 34).

The number of tillers increased up to panicle initiation and thereafter flowering stage a slight increase was observed. The treatment T4 - *Serratia* sp. (CB2) (9.00±0.34) registered more number of tillers at all the stages of growth. However the treatment T6, T8 and T11were on par with T4 at tillering stage. At panicle initation stage higher numbers of tillers were recorded in T4 -*Serratia* sp. (20.14±0.78) and T11 (19.60±1.35). The least number of tillers were observed with the treatment T12 (absolute control) at all the growth stages of observation (Table 35).

Table 34. Effect of inoculation of diazotrophic isolates on plant height (cm) of rice (cultivar-ADT43) at different growth stages

Isolate	Plant height (cm)		
	Tillering	Panicle initiation	Maturity
T1-*Klebsiella* sp.(CG1)+75% RDF	54.30 (± 4.32)^{a}	91.43 (± 8.53)^{a}	120.04 (± 4.78)^{a}
T2-*Bacillus* sp. (CG5)+75% RDF	49.40 (± 2.94) ^{ab}	80.24 (± 3.63) ^{b}	113.04 (± 3.45)^{b}
T3- *S. marcescens* (CD1)+75% RDF	48.43 (± 5.27) ^{ab}	78.35 (± 5.26)^{bc}	103.96 (± 2.56)^{d}
T4- *Serratia* sp. (CB2)+75% RDF	58.54 (± 2.27) ^{a}	94.46 (± 4.96)^{a}	118.84 (± 7.89)^{ab}
T5- *K. pneumoniae* (CR2)+75% RDF	49.56 (± 3.42)^{ab}	76.25 (± 5.68)^{bc}	112.76 (± 8.97)^{b}
T6- *Serratia* sp.(OR3)+75% RDF	47.67 (± 2.99) ^{ab}	79.23 (± 4.56)^{bc}	114.94 (± 6.78)^{b}
T7- *Serratia* sp. (CRE9)+75% RDF	48.75 (± 4.99) ^{ab}	84.29 (± 6.90)^{b}	106.72 (± 8.67)^{c}
T8- *Pseudomonas* sp. (CRE10)+75% RDF	56.15 (± 5.85)^{a}	92.30 (± 6.09)^{a}	123.83 (± 4.67)^{a}
T9- *P. agglomerans* (ORE9)+75% RDF	53.35 (± 3.85) ^{a}	91.53 (± 3.45)^{a}	110.68 (± 3.45)^{c}
T10- *A.lipoferum* (Az 204)*+75%RDF	51.43 (± 3.70) ^{a}	84.52 (± 5.46)^{b}	110.87 (± 2.56)^{c}
T11-100%RDF	53.62 (± 1.77) ^{a}	87.51 (± 3.45)^{b}	109.63 (± 8.90)^{c}
T12-Control (Absolute control)	40.53 (± 4.26)^{c}	73.62 (± 4.67)^{c}	101.82 (± 5.78)^{d}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); RDF- Recommended Dose of Fertilizer; *standard bioinoculant

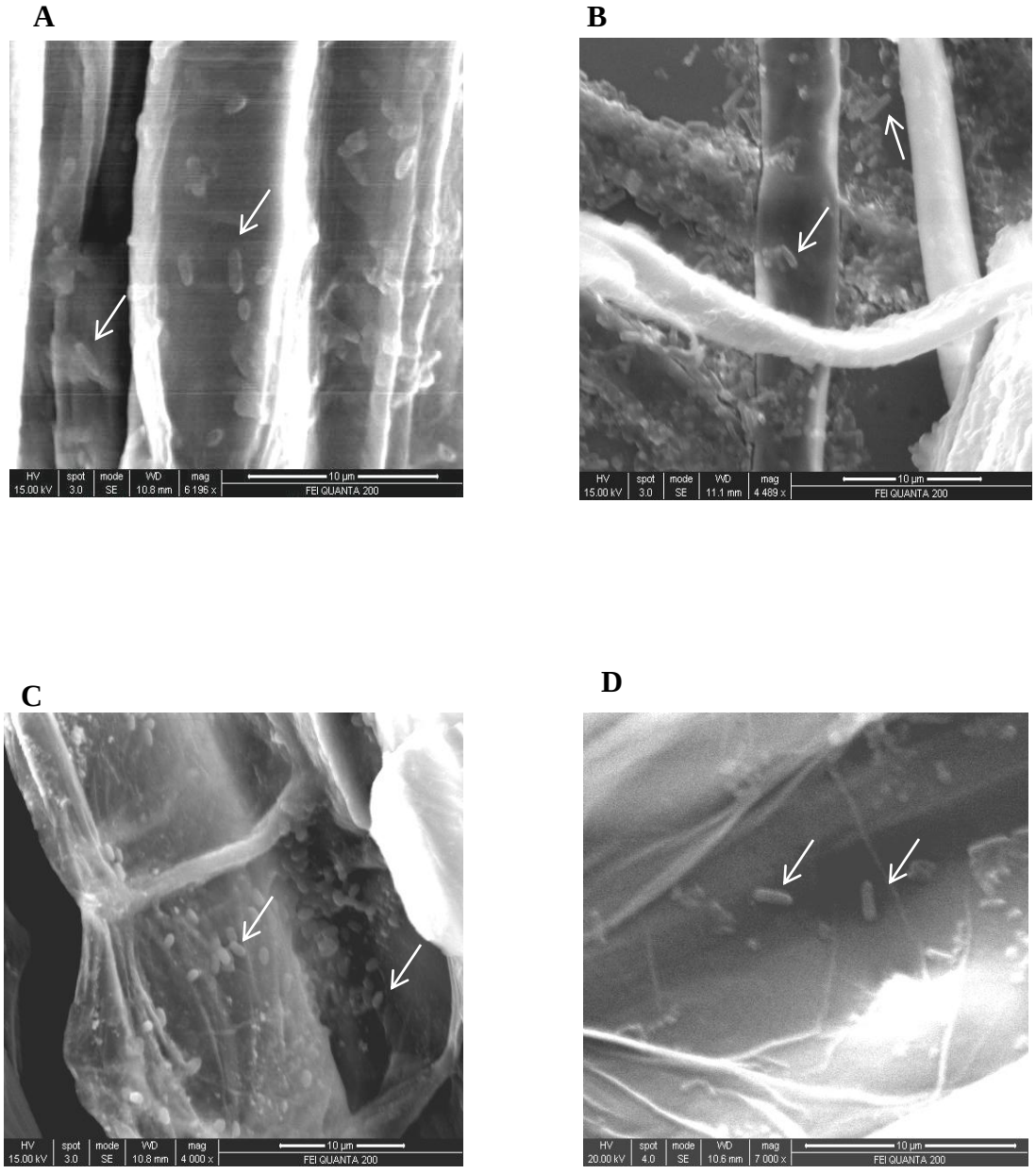

Plate 7a. Colonization of diazotrophic bacterial strain (*Serratia* sp. CRE9) on rice seedlings.
A- Longitudinal section of culm; B- Surface of the root; C,D- Longitudinal section of root. Arrow heads show the presence of bacteria

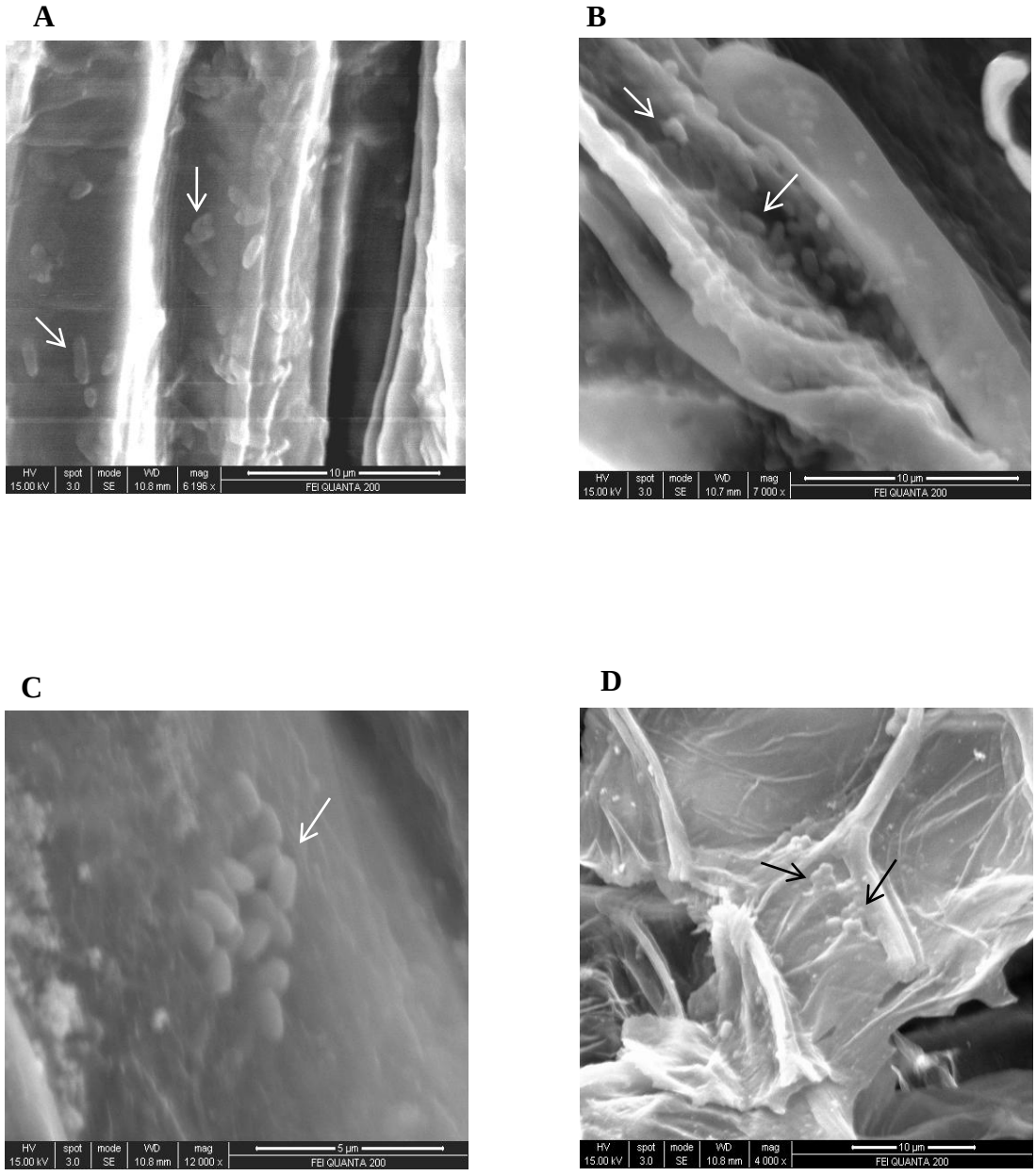

Plate 7b. Colonization of diazotrophic bacterial strain (*P. agglomerans* - ORE9) on rice seedlings. A- longitudinal section of culm; B- surface of the root; C,D- longitudinal section of root. Arrow heads show the presence of bacteria

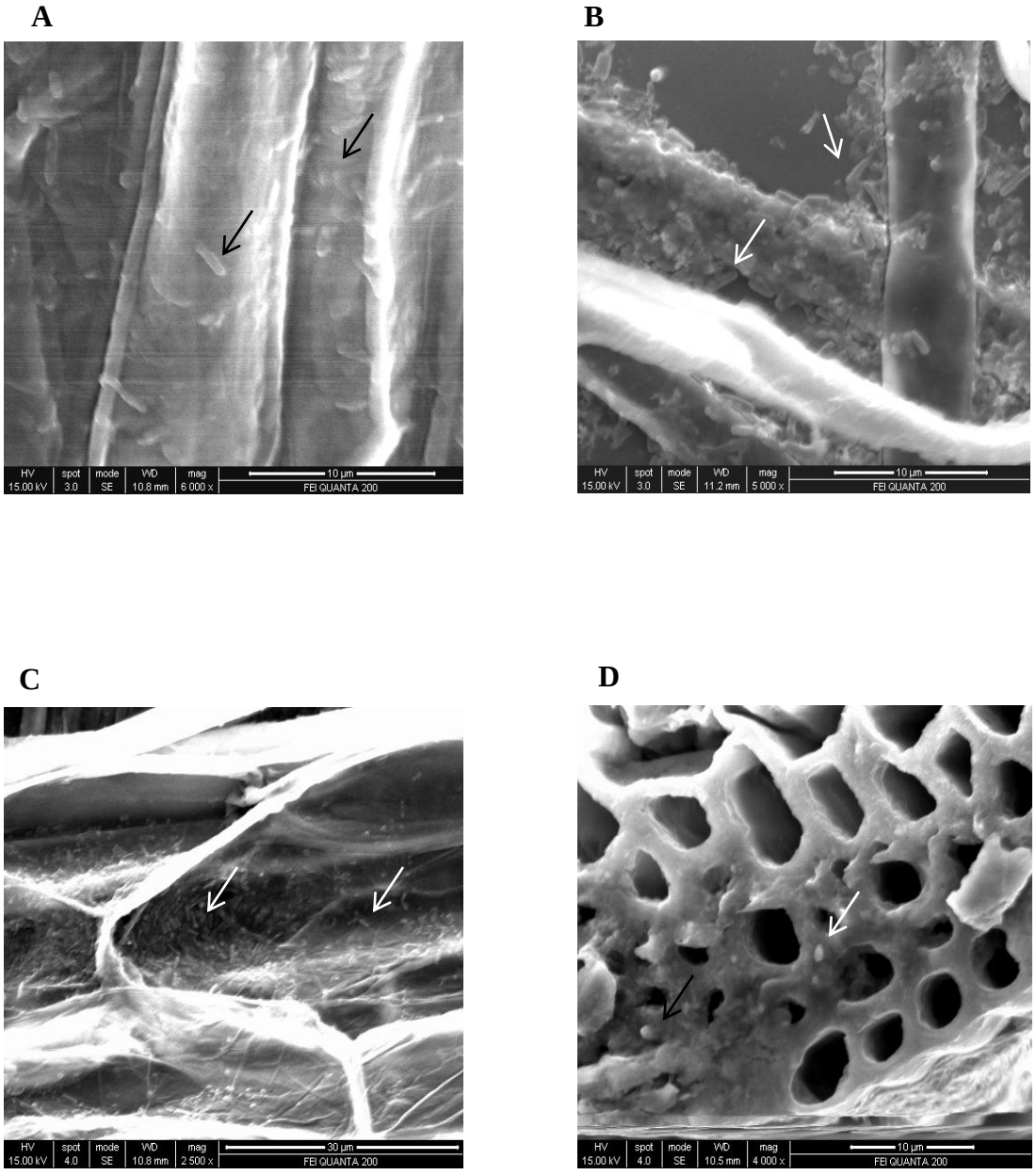

Plate 7c. Colonization of diazotrophic bacterial strain (*Pseudomonas* sp. CRE10) on rice seedlings. A- longitudinal section of culm; B- surface of the root; C- longitudinal section of root; D- transversal section of root. Arrow heads show the presence of bacteria

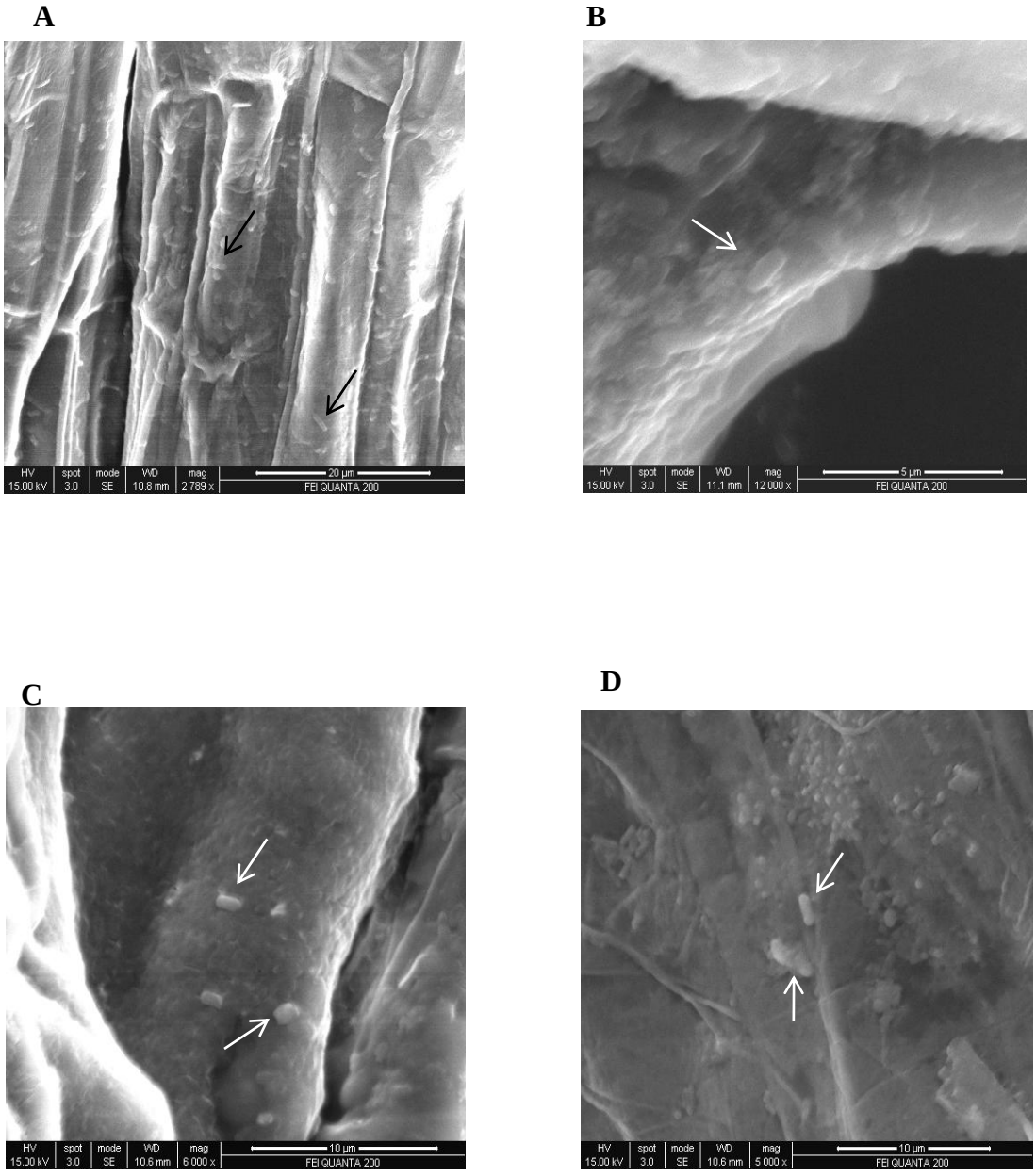

Plate 7d. Colonization of diazotrophic bacterial strain (*Enterobacter* sp. ORE7) on rice seedlings. A- longitudinal section of culm; B- surface of the root; C,D- longitudinal section of root. Arrow heads show the presence of bacteria

Plate 8. View of field experiment to evaluate the efficiency of diazotrophic isolates on rice (cultivar- ADT 43)

4.12.2. Effect of diazotrophic isolates inoculation on yield attributes, soil enzyme activity and N uptake

Application of diazotrophic isolates had a marked influence on number of effective tillers during the crop growing stages, were the maximum number (20.8±0.81) was observed in T4 - *Serratia* sp. while, the least was observed in treatment T12 (absolute control) (8.0±0.31) (Table 36). The panicle length and test weight were not significantly influenced by application of diazotrophic isolates (Table 36).

Application of diazotrophic isolates had significant influence on the grain yield (kg ha^{-1}) and the treatment T5 - *K. pneumoniae* (4997.72±344 kg ha^{-1}) recorded higher grain yield which was on par with T4- *Serratia* sp. (4967.64 ± 192.81kg ha^{-1}) while, the least grain yield was obtained in the absolute control (T12) (3034.90 ±118.56 kg ha^{-1}) whereas, application of diazotrophic isolates exhibited not significant difference in straw yield (kg ha^{-1}) (Table 37).

The higher amount of FDA was produced in the treatment T4- *Serratia* sp. (CB2) (18.86 ± 0.11 µg fluorescein g^{-1} h^{-1}) at maturity stage followed by T5- *K. pneumoniae* (CR2) (Fig. 10A and 10B). At maturity stage, maximum amount of ADA was recorded in the treatment T5 - *K. pneumoniae* 8.67±1.12 µg ammonia released g^{-1} soil h^{-1} followed by T8- *Pesudomonas* sp. (8.33±0.10 µg ammonia released g^{-1} soil h^{-1}) (Fig. 10A and 10B). Results showed that application of diazotrophic isolates made significant impact on uptake of nitrogen that was more in T4 - *Serratia* sp. (78.45 ± 3.04 kg ha^{-1}), while the next best treatment was T5 - *K. pneumoniae* (72.56 ± 5.01 kg ha^{-1}). The lowest nitrogen uptake was registered with treatment T12 (absolute control) (40.04±1.55 kg ha^{-1}) (Fig. 11).

Table 35. Effect of inoculation of diazotrophic isolates on number of effective tillers of rice (cultivar-ADT43) at different growth stages

Isolate	Number of tillers		
	Tillering	Panicle initiation	Maturity
T1-*Klebsiella* sp. (CG1)+75% RDF	4.90 (± 0.38)^{bc}	19.45 (± 1.55)^{ab}	20.42 (± 1.26)^{ab}
T2-*Bacillus* sp. (CG5)+75% RDF	5.45 (± 0.32)^{bc}	17.34 (± 1.03)^{ab}	18.10 (± 1.36)^{ab}
T3- *S. marcescens* (CD1)+75% RDF	6.34 (± 0.68)^{b}	18.23 (± 1.98)^{ab}	19.01 (± 2.13)^{ab}
T4- *Serratia* sp. (CB2)+75% RDF	9.00 (± 0.34)^{a}	20.14 (± 0.78)^{a}	20.48 (± 1.23)^{a}
T5- *K. pneumoniae* (CR2)+75% RDF	6.23 (±0.42)^{b}	18.46 (± 1.27)^{ab}	19.21 (± 1.45)^{ab}
T6- *Serratia* sp.(OR3)+75% RDF	8.34 (± 0.53)^{a}	19.23 ±1.20)^{ab}	19.47 ± 2.23)^{ab}
T7- *Serratia* sp. (CRE9)+75% RDF	4.00 (± 0.50)^{c}	15.13 (± 1.55)^{bc}	16.41 (± 2.34)^{bc}
T8- *Pseudomonas* sp. (CRE10)+75% RDF	8.45 (± 0.40)^{a}	18.46 (± 1.92)^{ab}	18.98 (± 1.89)^{ab}
T9- *P. agglomerans* (ORE9)+75% RDF	6.34 (± 0.88)^{b}	13.24 (± 0.82)^{c}	14.12 (± 1.86)^{c}
T10- *A. lipoferum* (Az 204)*+75%RDF	4.24 (± 0.31)^{c}	12.25 (± 0.72)^{c}	12.61 (± 1.74)^{c}
T11-100%RDF	8.50 (± 0.58)^{a}	19.60 (± 1.35)^{a}	19.78 (± 1.46)^{a}
T12-Control (Absolute control)	4.25 (± 0.16)^{c}	18.56 (±0.92)^{ab}	18.98 (± 1.96)^{ab}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); RDF- Recommended dose of fertilizer;*standard bioinoculant

Table 36. Effect of inoculation of diazotrophic isolates on yield attributes of rice (cultivar-ADT43)

Isolate	No of effective tillers	Panicle length (cm)	Thousand grain wt (g)
T1-*Klebsiella* sp.(CG1)+75% RDF	16.8 (± 1.34)^{bc}	26.5 (± 2.11)^{a}	18.9 (± 1.50)^{b}
T2-*Bacillus* sp. (CG5)+75% RDF	18.6 (± 1.11)^{ab}	23.7 (± 1.41)^{a}	19.3 (± 1.97) ^{b}
T3- *S. marcescens* (CD1)+75% RDF	18.7 (± 2.03)^{ab}	26.3 (± 2.86)^{a}	18.1 (± 0.70)^{b}
T4- *Serratia* sp. (CB2)+75% RDF	20.8 (± 0.81)^{a}	27.9 (± 1.08)^{a}	20.6 (± 2.30) ^{b}
T5- *K. pneumoniae* (CR2)+75% RDF	19.8 (± 1.37)^{ab}	27.8 (± 1.92)^{a}	29.4 (± 1.23)^{a}
T6- *Serratia* sp.(OR3)+75% RDF	18.8 (± 1.18)^{ab}	25.4 (± 1.59)^{a}	19.7 (± 1.03) ^{b}
T7- *Serratia* sp. (CRE9)+75% RDF	12.6 (± 1.29)^{d}	22.6 (± 2.32)^{a}	18.9 (± 2.03)^{b}
T8- *Pseudomonas* sp.(CRE10)+75% RDF	13.8 (± 1.44)^{cd}	25.8 (± 2.69)^{a}	19.5 (± 1.19)^{b}
T9- *P.agglomerans* (ORE9)+75% RDF	14.0 (± 0.87)^{cd}	24.8 (± 1.54) ^{a}	19.2 (± 1.43)^{b}
T10- *A. lipoferum* (Az 204)*+75%RDF	14.0 (± 1.05)^{cd}	25.8 (± 1.93) ^{a}	19.1 (± 1.30)^{b}
T11-100%RDF	18.0 (± 1.24)^{ab}	24.6 (± 1.70)^{a}	18.8 (± 0.09)^{b}
T12-Control (Absolute control)	8.0 (± 0.31)^{e}	23.4 (± 0.91)^{a}	16.8 (± 0.65)^{b}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); RDF- Recommended Dose of Fertilizer;*standard bioinoculant

Table 37. Effect of inoculation of diazotrophic isolates on yield of rice (cultivar-ADT43)

Isolate	Grain yield (kg ha^{-1})	% increase over control	Straw yield (kg ha^{-1})	% increase over control
T1-*Klebsiella* sp. (CG1)+75% RDF	3456 (± 275.08)^{c}	-8.7	3874 (± 308.32)^{a}	8.2
T2-*Bacillus* sp. (CG5)+75% RDF	3897 (± 232.25)^{bc}	2.8	3890 (± 231.82)^{a}	8.1
T3- *S. marcescens* (CD1)+75% RDF	4012 (± 436.60)^{bc}	5.9	3906 (± 425.01)^{a}	8.5
T4- *Serratia* sp. (CB2)+75% RDF	4967 (± 192.81)^{a}	31.0	3986 (± 154.74)^{a}	10.8
T5- *K. pneumoniae* (CR2)+75% RDF	4997 (± 344.75)^{a}	32.0	3945 (± 272.19)^{a}	9.6
T6- *Serratia* sp. (OR3)+75% RDF	4645 (± 290.96)^{ab}	22.6	3784 (± 236.99)^{a}	5.1
T7- *Serratia* sp. (CRE9)+75% RDF	3986 (± 369.37)^{bc}	5.0	3689 (± 377.96)^{a}	2.5
T8- *Pseudomonas* sp. (CRE10)+75% RDF	3545 (± 234.73)^{c}	-6.4	3478 (± 362.41)^{a}	-3.4
T9- *P. agglomerans* (ORE9)+75% RDF	3234 (± 261.40)^{c}	-14	3567 (± 221.21)^{a}	-0.8
T10- *A. lipoferum* (Az 204)*+75%RDF	3134 (± 129.23)^{c}	-17	3445 (± 257.97)^{a}	-4.2
T11-100%RDF	3789 (± 122.68)^{bc}	-	3598 (± 275.85)^{a}	-
T12-Control (Absolute control)	3034 (± 118.56)^{c}	-19	3016 (± 126.43)^{b}	-16

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT ($p \leq 0.05$); RDF- Recommended Dose of Fertilizer;*standard bioinoculant

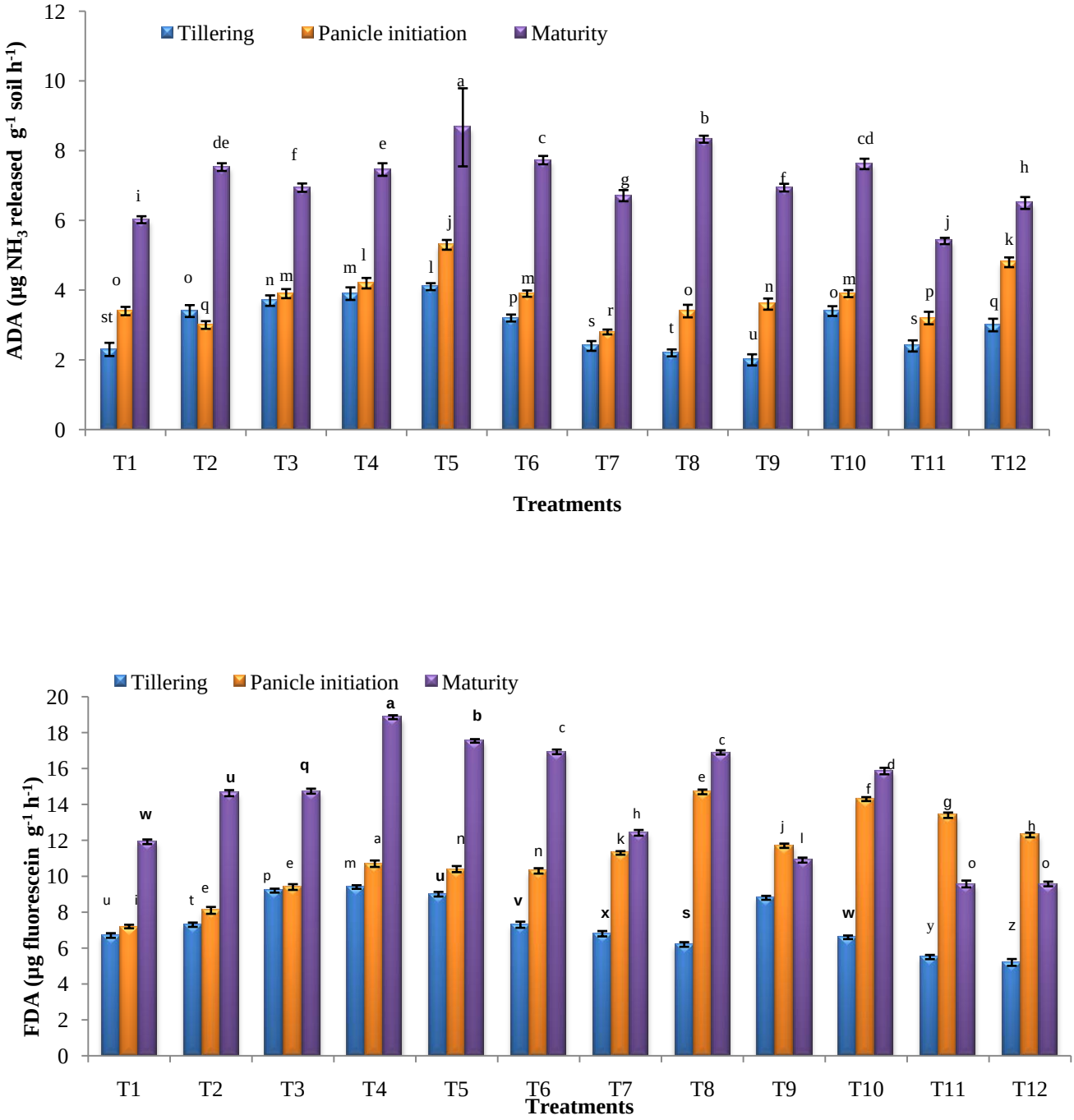

Fig 10. Effect of inoculation of diazotrophic isolates on soil enzymes, arginine deaminase activity (A) and fluorescein diacetate activity (B) at different stages of rice cultivation. Data are presented as mean ± SE (n=3); For each panel, different letters indicate significantly different at P≤0.05 according to Duncan's Multiple Range Test. T1-T12: Treatments enforced as detailed in table 38.

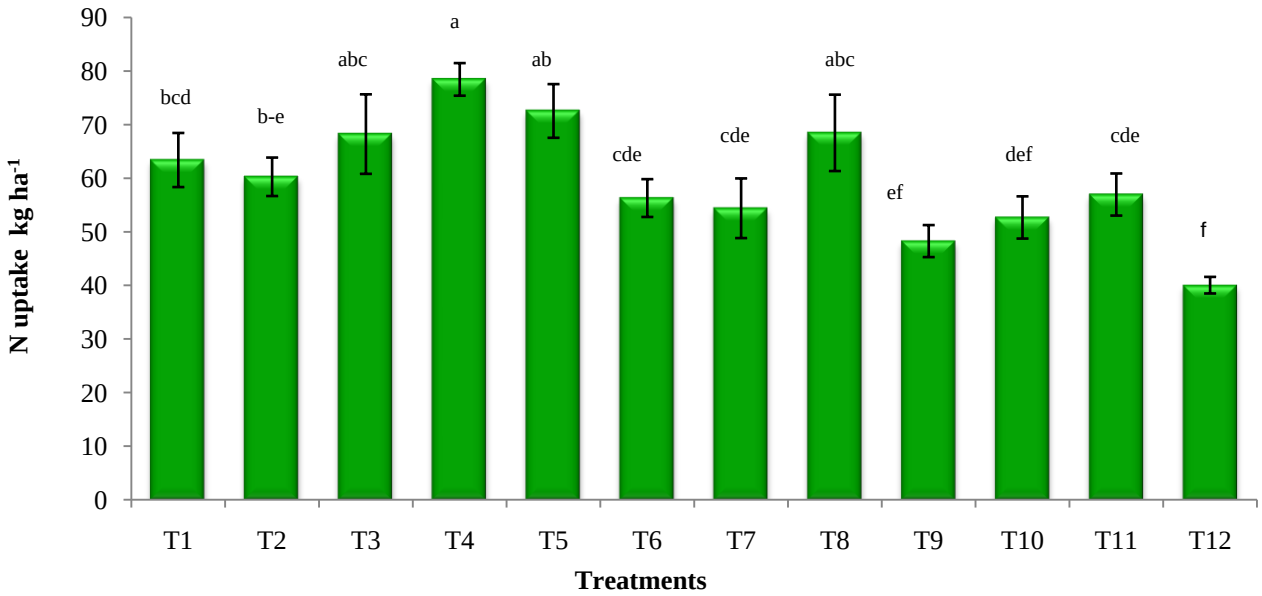

Fig 11. Effect of inoculation of diazotrophic isolates on N uptake of rice. Data are presented as mean ± SE (n=3); For each panel, different letters indicate significantly different at P≤0.05 according to Duncan's Multiple Range Test. T1-T12: Treatments enforced as detailed in table 38.

Discussion

CHAPTER V

DISCUSSION

Microbes have been shown to be important partners in mitigating the effect of virtually every known environmental stress that can affect the plant (Reid and Greene, 2012). Enrichment of soil with diazotrophs possessing useful plant growth-promoting (PGP) traits aids in the reduction of nitrogenous - fertilizer requirement and thereby the fertilization costs as well as minimizing the risk of nitrate pollution. Plant-associated bacteria play key roles in their host's adapting to changing environments in various ecosystems. These interactions between plants and beneficial bacteria can significantly affect the general plant health and soil quality. In legumes, plant specific enrichment of microflora in the rhizosphere milieu has been exploited under nitrogen limiting conditions. Likewise, non-leguminous crops select specific bacterial groups in the rhizosphere (Lemanceau, 1995). Studies predict the possible role of diazotrophic bacteria in maintaining soil fertility and N input in disturbed ecosystems (Purushothaman *et al*., 1980 and Barraquio *et al*., 1997). Apart from soil bacteria several endophytic bacteria enhance growth and improve general plant health (Stoltzfus *et al*., 1997).

Hence, the present investigation was carried out to isolate culturable diazotrophic bacteria associated with ten selected grass species, which are normally grown in various physiographic regions of India. These diazotrophs were screened for high nitrogen fixation and the isolates were identified based on 16S rRNA gene sequencing. Further, the multiple plant growth promoting traits and tolerance level to various abiotic stress conditions were examined. The influence of diazotrophic bacterial inoculation on growth of rice was also studied both under *in vitro* and *in vivo* conditions. The results obtained from these experiments are discussed in this chapter.

5.1. Physico-chemical and biological properties of the soils hosting the grass species

The rhizosphere is the narrow region of soil influenced by the root exudates and associated soil microorganisms. The rhizosphere region is a highly favourable habitat for the proliferation, functioning and metabolic activity of numerous microorganisms. It is characterized by greater microbiological activity near rhizosphere than the soil away from plant roots (Berg *et al*., 2002). Rhizodeposits make the rhizosphere a desirable niche for

microbial communities to proliferate. A better understanding of these processes is critical for maintaining the health of plant and feeding the organism that live on it (Moorissy *et al*., 2004). The results of the present study confirmed that the rhizosphere had predominant influence on physico-chemical as well as biological properties of the soil. It is now established that greater number of bacteria, fungi, actinobacteria and diazotrophs are present in the rhizosphere soil than in bulk soil and there are innumerable reports in literature to substantiate this fact. The present study also revealed higher Fluorescein diacetate (FDA) hydrolysis and arginine ammonification rates in the rhizosphere soils and found to have positive correlation with soil microbial activity. FDA hydrolysis rates have been used to estimate total microbial activity (Bandick and Dick 1999 and Schnurer and Rosswall, 1982). FDA is a substrate for a wide variety of enzymes, including proteases, lipases, and esterases. Several factors such as soil type, moisture, pH and temperature and the age and condition of plants are known to influence the rhizosphere effect. Plant genotypes differ in their capacity to convert non-available forms of nutrients to available forms and to take them up. Factors underlying the differential capacities of plant genotypes to access soil nutrients include differences in the surface area of contact between roots and soil (Sadana *et al*., 2002) and in the composition and amount of root exudates (Jones *et al*., 2004) and rhizosphere microflora, resulting in differences in the chemistry and biology of the rhizosphere. The nutrient availability in the rhizosphere is controlled by the combined effects of soil properties, plant characteristics and the interaction of roots with microorganisms (Jones *et al*., 2004). The concentration of nutrients and their availability to plants differ between the rhizosphere and the bulk soil (Marschner, 1995). Nutrient availability for microbial growth is higher in rhizosphere compared to non rhizosphere soil.

Fluorescein diacetate hydrolysis rate and arginine ammonification are the widely accepted accurate and simple methods for measuring total microbial activity in aerobic soils. Because they are mediated simultaneously by protease, esterase and lipase and thereby reflect the activities of these enzymes in soil (Adam and Duncan, 2001 and Alef and Kleiner, 1986). Since 90% of energy flow in a system passes through microbial decomposers and heterotrophic microorganisms are predominant in soil, FDA hydrolysis is thought to reflect overall soil microbiological activity (Schnurer and Rosswall, 1982). Similar dynamic trend is also revealed in the determination of total soil microbial activity as shown in Fig.4. In the present study, 50% of soils

from selected grass species were saline and recorded lower enzyme activity which revealed that arginine ammonification rate and FDA hydrolysis rate are all negatively correlated with salinity. A Similar study showed that, salt affected arid soils in China have lower FDA hydrolysis rate and arginine ammonification rate (Rietz and Haynes, 2003).

5.2. Richness of rhizospheric and endophytic diazotrophs in grass species

Different diazotrophic growth media such as N-Free malate medium, JNFb, LGI, SSM, TDM, SM and Rennie medium were used to isolate the diazotrophic bacterial species. The results showed that, 53% of rhizosphere diazotrophic population appeared on N-Free malate medium without addition of NH_4Cl as a nitrogen source similar result has been also reported by Sgroy *et al.* (2009). The endophytic isolations were made in nitrogen-free semisolid media with different carbon sources and pH values. This semisolid nitrogen free media offer the possibility for diazotrophic bacteria to find the right niche for nitrogen fixation. In the present study, 33% of endophytes were obtained by using N-free modified Rennie medium. The inclusion of N-free modified Rennie medium for isolation of diazotrophs turned out to be very useful, as the endorhizosphere bacteria could only be detected on this medium, indicating that these bacteria may be adapted to high salt concentrations. The present finding are in agreement with Barraquio *et al*. (1997) and Elbeltagy *et al.* (2001) who reported on the isolation of nitrogen fixing bacteria from N-free media. To test the endophytic nature of bacteria, experiments were performed to fulfill Koch's postulates and to examine their endophytic competence (infection and persistence characteristics). The results indicated that 61% of diazotrophic endophytic bacteria could be isolated from the selected grass tissues, which were capable of re-colonizing their host when re-inoculated onto sterile rice seedlings.

Twenty seven percent of endophytic isolates showed both pectinase and cellulase producing ability which are required for penetration and spread inside the plant. Pectinase, is a key enzyme for colonization. Pectinolytic activity has been proposed to be responsible for root invasion by *Azospirillum* spp., through middle lamellae and points of emergence of lateral roots (Patriquin *et al*., 1983).

For active penetration, endophytic bacteria have to be well-equipped with cellulolytic enzymes which hydrolyze the plant's exodermal cell walls. *In vitro* production of these enzymes has been reported for many endophytes (Compant *et al*., 2005 and Reinhold-Hurek *et al*., 2006). The expression of endoglucanase, the main cellulase responsible for hydrolysis of β (1-4) linkage in cellulose, is detected at the primary sites of entry of *Azoarcus* sp. BH72 (Reinhold-Hurek *et al*., 2006). By entering a plant through natural cracks at the region where the lateral roots appear, bacteria remain "invisible" for the plant's immune system. This mode of entry (often combined with active penetration) has been suggested for *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek, 1998) and *Burkholderia vietnamiensis* (Govindarajan *et al*., 2008) in rice, *B. phytofirmans* PsJN in grape (Compant *et al*., 2005) and *Gluconacetobacter diazotrophicus* in sugar cane (James *et al*., 1994) and *Herbaspirillum seropedicae* Z67 in rice (James *et al*., 2002). Furthermore, Bekri *et al*. (1999) mentioned that besides gaining entry into the plant through natural openings and wounds, endophytic bacteria actively penetrate plant tissues using hydrolytic enzymes like cellulase and pectinase.

5.3. Nitrogen fixing potential of diazotrophs obtained from selected grass species

The diazotrophic isolates were grown in chemically defined nitrogen-free medium. The results showed 61% of rhizosphere isolates and 95% of endophytic diazotrophs are recorded positive growth in N- free medium and this capability could be attributed to the acquisition of atmospheric nitrogen by biological fixation. Doty *et al*. (2009) reported a high percentage of the diazotrophic endophytes from the native black cottonwood and willow, are able to grow on this nitrogen-free medium. In another work, Forchetti *et al*. (2007) isolated endophytic *Achromobacter xylosoxidans* and *Bacillus pumilus* from sunflower (*Helianthus annuus* L.) roots and found both are capable of growing in chemically defined medium without nitrogen source. Gyaneshwar *et al*. (2001) characterized nitrogen fixation by using nitrogen-free dextrose medium and isolated the endophytic diazotrophic strain *Serratia marcescens* from rice.

In similar experiments, free-living nitrogen fixing *Lysinibacillus fusiformis* isolated from rhizosphere of different crops exhibited highest nitrogenase activity in controlled experimental conditions (Park *et al*., 2005). The nitrogen fixing abilities of *B. subtilis*,

B. pumilus, *Brevibacterium halotolerans* and *P. putida* have been revealed by growing them in nitrogen free medium, as qualitative evidence of atmospheric nitrogen fixation (Sgroy *et al*., 2009). The isolates showing growth in N- free medium were further subjected to ammonia production and analysed for total nitrogen content. The results showed that 75% of rhizosphere and 95% endophytic diazotrophic isolates produced ammonia and total nitrogen content. Similarly, Barua *et al*. (2012) reported that all the diazotrophic isolates from saline habitats of sunderbans fixed the dinitrogen. Islam *et al*. (2009) reported ammonia production in all diazotrophs isolated from paddy fields.

The discovery that the nitrogenase enzyme responsible for N_2-fixation also reduced C_2H_2 (acetylene) to C_2H_4 (ethylene) (Dilworth, 1966) provided a useful tool for the assessing N_2-fixation process. In the present study, all the selected diazotrophs were subjected to acetylene reduction assay, of which, 57% of rhizosphere diazotrophic isolates and 42% endophytic diazotrophic isolates recorded acetylene reduction. The rest recorded no acetylene reduction activity. Similar to our results, Ribbe *et al*. (1997) reported that, microbes that could grow in nitrogen-free medium yet were negative in the acetylene reduction assay. One possible explanation is that the test conditions may not be optimized for these isolates even though other isolates showed acetylene-reducing activity under the same conditions. Brighnigna *et al*. (1992) demonstrated that some epiphytic isolates could grow in nitrogen-free medium although they were acetylene reduction negative.

Differences in the composition of culture media and incubation time may have led to the contradictory results on nitrogenase activity in pure culture. Han and New (1998) reported that *Azospirillum* isolates had a wide range of nitrogenase activity (0.0-154.9 n moles ethylene mg^{-1} protein h^{-1}) while Malik *et al*. (1997) found a high nitrogenase activity (686 n moles ethylene mg^{-1} protein h^{-1}). Venieraki *et al*. (2011) reported the acetylene reduction activity ranged from 4.5±0.2 to 44.3±5.7 nmoles ethylene mg^{-1} protein h^{-1} for the 11 isolates from field grown barley, oat and wheat.

The acetylene reduction of pure culture is commonly performed with a favourable carbon source and the evidences in this study which showed that most isolates were obtained from different growth media. The low nitrogenase activity in this work thus might be due to an inappropriate carbon source.

In the present study the rhizosphere diazotrophic isolate OR3 (*Serratia* sp.) and endophytic isolate CGE3 (*Pseudomonas* sp.) showed increased activity (250% and 263% respectively) when compared to acetylene reduction by the standard culture. Islam *et al*. (2009) isolated free-living diazotrophic bacterium *Serratia* sp. which has appreciably high nitrogenase activity (115 n moles ethylene mg^{-1} protein h^{-1}) from paddy fields. Diazotrophy in *Serratia* sp. was confirmed on the basis of nitrogenase activity which produced 12.5 nmol of ethylene h^{-1} 10^6 $bacteria^{-1}$ (Gyaneshwar *et al.*, 2001).

Mirza *et al*. (2006) isolated a marine *Pseudomonas alcaligenes* having potential of diazotrophy and good supply of nitrogen to rice plants when inoculated in non-sterile soils. Similar diazotrophy has also been reported earlier in *Pseudomonas stutzeri* isolated from rice roots. Similarly, Barraquio and Watanabe (1983) reported that nitrogen fixing and hydrogen utilizing *Pseudomonas* sp. was predominant in wetland rice roots.

5.4. Diversity of diazotrophs isolated from grass species

5.4.1. Metabolic diversity

The presence of diverse groups of nitrogen-fixing bacteria was established by assessing various morphological characteristics of the diazotrophic isolates from selected grass species. The morphological characters such as cell shape, colony morphology and Gram staining revealed the presence of diverse groups of bacteria. The results of Gram reaction disclosed that 83% diazotrophic bacteria were Gram negative and remaining 17% are Gram positive. Zinniel *et al*. (2002) obtained almost equal distribution of Gram positive and Gram negative isolates from maize and sorghum.

The carbon substrate utilization profile of the isolates, used in this study also exhibited high divergence among the isolates. The data obtained from this profiling were subjected for cluster analysis of the diazotrophs. The 24 diazotrophic isolates from rhizosphere clustered into 7 different groups. The differences in the ability to utilize various carbon sources may be due to the presence or absence of specific operons and structural genes required for the breakdown of the specific carbon substrate. Clustered and principal component analysis of BIOLOG data also used the designation of 8 physiologically distinct strain groupings from salt marsh grass study by Bagwell *et al*., (2013). Similarly, the tests on antibiotic resistance showed that high proportion of the diazotroph isolates intrinsically

resistant to the 10-12 antibiotics tested (Bagwell *et al*., 2013). Based on the result the 24 isolates are clustered into 7 different groups. Gilbert *et al*. (1993) also found a high proportion of rhizosphere bacteria resistant to antibiotics. PGPR bioinoculants resistant to high level of antibiotics might have ecological advantage for survival in the rhizosphere.

In the present study, diazotrophs *Klebsiella* sp., *B. licheniformis*, *Serratia* sp., and *Pseudomonas* sp. were found to be resistant to more number of antibiotics than other isolates. Evidence of increasing resistance to antibiotics in soil and other natural isolates highlights the importance of horizontal transfer of resistance genes in facilitating gene flux in bacteria. Horizontal gene transfer in bacteria is favoured by the presence of mobile genetic elements and by the organization of bacterial genomes into operons allowing for the cooperative transfer of genes with related functions. For example, Nathisuwan *et al*. (2008) reported that *Klebsiella* with the ability to produce extended-spectrum beta-lactamases, possessed resistance to many classes of antibiotics.

5.4.2. Molecular diversity

Identification of new isolates based on phenotypical and physiological criteria however is difficult, if the features displayed by a particular isolate are not fully identical with a described species. Based on16S rRNA gene sequence analysis, the diazotrophic bacteria found in the selected grasses belonged to Enterobacteriaceae (75%), Bacillaceae (16%) and Pseudomonaceae (8%).

Nearly 20% of diazotrophic isolates showed similarity to *Klebsiella* sp. and 16% of isolates closely resembled *K. pneumoniae*. Chelius and Triplett (2000) reported that *K. pneumoniae* as an endophyte in maize. In wheat, Iniquez *et al*. (2004) demonstrated and confirmed the nitrogen fixing activity of *K. pneumoniae*. The nitrogen fixing activity of *K. pneumoniae* isolates were again confirmed by our work. Among the diazotrophic isolates *Serratia* sp. accounted for 12% of which, 4% of isolates belonged to *S. marcescens*. Diverse species of *Serratia* have been isolated from cotton and sweet corn (McInroy *et al*., 1995), rice rhizosphere (Rosales *et al*., 1993) rice seed (Mukhopadhyay *et al*., 1996). *Serratia* has been found as an endophytic colonizer of rice (Gyaneshwer *et al*., 2001). Giudice *et al*. (2008) reported non-pigmented *S. marcescens* from vetiver (*Vetiveria zizanioides).*

Both these findings are similar to current findings that non-pigmented *S. marcescens* are prevalent in grass species. However, the most predominant and studied endophytes belong to three major phyla (Actinobacteria, Proteobacteria and Firmicutes) and include members of *Azoarcus* (Krause *et al*., 2006), *Gluconobacter* (Bertalan *et al*., 2009), *Bacillus* (Deng *et al*., 2011), *Enterobacter* (Taghavi *et al*., 2009), *Burkholderia* (van *et al*., 2000), *Herbaspirillum* (Pedrosa *et al*., 2009), *Pseudomonas*, *Serratia* (Taghavi *et al*., 2009), *Stenotrophomonas* (Ryan *et al*., 2009) and *Streptomyces* (Suzuki *et al*., 2005).

In the present work, firmicutes were mainly dominated by different groups of *Bacillus*, which have been isolated from selected grass species is in accordance with the findings of Chowdhury *et al*. (2009). Approximately 75% of the firmicutes were able to form endospores indicating the capacity to deal with environmental stress. Additionally, a similar quantity of isolates was assigned to the phylum Actinobacteria, which includes many common soil bacteria that are well adapted to environmental fluctuations such as desiccation. For instance, four isolates were identified as *Arthrobacter* and two as *Microbacterium*, that are known to persist drought periods by forming 'resting cocci' (Goodfellow and Williams, 1983) or highest level of desiccation tolerance, or may even promote drought-stress tolerance to plants (Narvez-Reinaldo *et al*., 2010). The endophytic strains such as *Pantoea* sp. and *P. agglomerans* were isolated from tissues of wild rice and *Panicum repens* respectively. Similar to our results, Whitton and Rother (1988) reported that three different types of *P. agglomerans* are the dominant members of the endophytic bacterial community present within the floating rice which have N fixing ability. Endophytic *Pantoea* are reported in sugarcane (Loiret *et al*., 2004) and in soybean (Kuklinsky-Sobral *et al*., 2004). The endophytic *Pseudomonas* sp. was isolated from tissues of *Cenchrus glaucus* and *Cyperus rotundus.* Behrendt *et al*. (1999) identified the *Pseudomonas graminas*, a yellow-pigmented, plant-associated bacterium from grasses. In the present investigation, *Enterobacter* sp. accounts for 8% of the total diazotrophs members of enterobateriales are known N_2-fixers and one of the most universal of endophytic genera. *Enterobacter* has been identified as endophytes of several plants such as *Citrus sinensis*, soybean, sweet potato and maize (Araújo *et al*., 2002; Zinniel *et al*., 2002 and Kuklinsky-Sobral *et al*., 2004). Doty *et al*. (2009) isolated diazotrophic endophytes *Pantoea* sp. and *Enterobacter* sp. from grasses. Among the diazotrophs, one isolate from

Saccharum spontaneum (SS4) identified as *Stenotrophomonas* sp. is ubiquitous and often associated with plants has reportedly been isolated from rhizosphere of grass, wheat, oat, cucumber, maize, oilseed rape, potato and lettuce (Park *et al*., 2005; Chowdhury *et al*., 2007). It is not yet defined as nitrogen fixing bacteria despite their nitrogen fixing activities have been investigated and confirmed (Liu *et al*., 2007). Cibichakravarthy *et al*. (2011) isolated the diazotroph *Stenotrophomonas maltophila* from the rhizosphere of *Prosopis*. Roots of *Spartina alterniflora*, a common smooth cordgrass growing in salt marsh of North America harboured several gamma proteobacterial diazotrophs (Bagwell *et al*., 1998; Bagwell and Lovell 2000).

5.4.3. *nifH* gene diversity

New molecular technology utilizing analysis of *nifH* gene, a structural gene for highly conserved nitrogenase protein, helped in identifiing previously unrecognised diazotrophic microorganism. The N_2 fixation requires the interaction of several gene products including the nitrogenase structural proteins like *nifD*, *nifK*, and *nifH*. The phylogeny based on *nifH* genes has been shown to resemble the 16S rRNA phylogeny (Zehr *et al*., 2003); thus *nifH* is an ideal phylogenetic gene marker for investigating N_2-fixing organisms in natural environments. In the past, *nifH* gene has been successfully used to determine diversity of the diazotrophic communities (Roesch *et al*., 2006; Albino *et al*., 2006; Wakelin *et al*., 2007).

The amplification of approximately 550 bp long *nifH* fragment was carried out using universal primers *nifH*-b1 and CDHPnif723R. The *nifH* genes could be amplified from 62% of isolates from rhizosphere and 55% of endophytes from different grass species. Our results are in agreement with earlier studies (Kuklinsky-Sobral *et al*., 2004, Chowdhury *et al*., 2007) where *nifH* gene could not be amplified because of the variability of this gene (Zehr *et al*. 2003). It has been reported that no direct correlation between the presence of *nifH* and the acetylene reduction activity of the bacterial strains (Dean and Jacobson, 1992). In this study the *nifH* amplification could be clearly detected in *Klebsiella* sp., *Enterobacter* sp., *B. subtilis, K. pneumoniae, Serratia* sp., *Bacillus* sp., S. *marcescens, Pseudomonas* sp. and *P. agglomerans*. Identification of nitrogen-fixing heterotrophic bacteria isolated from rice fields in the Yangtze River Plain by Xie *et al*. (2006)

showed the existence of *nif* genes in three strains of *Bacillus* sp. Ando *et al*. (2005) detected *nifH* DNA genes in *Klebsiella* sp., and *Serratia* sp. isolated from mature sugarcane harvested on Tanegashima and Miyako Islands, Japan. DNA sequence analysis demonstrated that the *nifH* gene sequences were highly similar to those from *K. pneumoniae.* It possesses a total of 20 *nif* genes that are clustered in a 24 kb region of the chromosome and are responsible for nitrogenase synthesis and regulation (Chelius and Triplett, 2000). The presence of *nifH* genes was confirmed by amplification in endophytic diazotrophic strain *Serratia marcescens* isolated from rice Gyaneshwar *et al*. (2001)

5.5. Plant growth promoting traits of diazotrophic bacteria from grass species

There has been increasing evidence that besides N_2–fixation, synthesis and export of phytohormones by the N_2–fixing bacteria may play an important role in plant growth promotion. Phytohormones also called plant growth regulators (PGR) are well known for their regulatory role in plant growth and development. Venieraki *et al*. (2011) reported all the 11 strains isolated from salt marsh grass exhibited both diazotrophic and IAA production to the tune of 18.4±5.4 to 194.8±17.1 µg IAA mg^{-1} protein. Muthukumarasamy *et al*. (2007) and Ahmad *et al*. (2008) reported that many strains of PGPR isolated from rhizosphere soils, rhizoplane or from inside plant tissues of Gramineae plants were found to have PGPR activity along with diazotrophy is agreement with our results. After establishing in a plant, endophytes can positively influence plant growth and its resistance to different stresses. It is likely that some diazotrophic bacteria stimulate plant growth both by supplying N and by production of phytohormones, in particular IAA. This possibility is further supported by the observation that when N was not limiting, both wild type *G. diazotrophicus* Pal 5 and its fix-mutant strains were able to increase the biomass of sugar cane (Sevilla *et al*., 2001).

Members of the Enterobacteriaceae isolated from selected grass species have been known to possess plant growth promoting traits, such as ability to fix nitrogen, produce IAA, GA and mineralize insoluble plant nutrients. They have earlier been also shown to be potent biological control agents against fungal diseases.

The production of IAA and related compounds has been demonstrated in many diazotrophs, including *Acetobacter diazotrophicus*, *Azospirillum* sp., *Azotobacter* sp., and

Paenibacillus polymyxa (Dobbelaere *et al*., 2003). The IAA increases plant metabolism and hence inoculation of diazotrophic IAA producing strains in saline soil is of immense importance, because in addition to increasing the nitrogen status of soil, it may promote plant growth (Alexander, 1977). The organisms exhibited both diazotrophic character and producer of IAA which make them good candidates for crop growth. Besides N fixation, the production of IAA and related compounds by *K. pneumoniae* in culture media supplemented with tryptophan was reported in our results in accordance with findings of El-Khawas and Adachi (1999). In addition, phytohormone production (Timmusk *et al*., 1999), mineral solubilizing (Vasquez *et al*., 2000 and Canbolat *et al*., 2006) and biocontrol properties (Kloepper *et al*., 2004 and McSpadden-Gardener, 2004) by different strains of *Bacillus* have also been widely reported and were again confirmed by our work. *P. agglomerans* is a known diazotrophic endophyte of rice, and has been shown not only to fix nitrogen but also produce phytohormones and promote plant growth (Verma *et al*., 2001). Our isolate *P. agglomerans* on endophyte of wild rice also shown maximum production (126%) of IAA. The *in vitro* production of IAA and its possible involvement in PGP has been reported for many other endophytic bacteria (Govindarajan *et al*., 2008). Our findings agree with previous reports that the most of the plant associated bacteria were able to produce a variety of plant growth promoting substances in considerable amounts apart from diazotrophy.

In nature, 1-aminocyclopropane-1-carboxylate deaminase has been commonly found in soil bacteria that colonize plant roots (Glick *et al*., 1999). The distribution of ACC deaminase activity is common among plant growth promoting bacterial groups. In the present study, 43% of rhizosphere diazotrophs (*B. subtilis, K. pneumoniae, Serratia* sp., *B. licheniformis*, *S. marcescens*, *Bacillus* sp.) and 55% of endophytic diazotrophs (*Serratia* sp., *Pseudomonas* sp., *Enterobacter* sp., *P. agglomerans*, *E. sacchari*) showed the ACC deaminase activity. Higher amount of ACC deaminase is produced by endophytic bacteria *P. agglomerans* (85%) in our study. Similar to our results, Teng *et al*. (2010) found that *P. agglomerans* isolated from rice possessed the ACC deaminase along with phosphate-solubilizing ability, siderophores and phytohormones production. Bacteria containing ACC deaminase bind to roots and/or seed coats and stimulate root elongation by lowering the ethylene level in plants. Many of these microorganisms are identified by their ability to grow on minimal medium containing ACC

as its sole nitrogen source. In this way, *Azospirillum* spp., *Herbaspirillum* spp., *Azoarcus*, *Azorhizobium caulinodans*, *Gluconacetobacter diazotrophicus*, *Burkholderia vietnamiensis*, *Azotobacter* spp., *Azorhizophilus* and *Pseudomonas* spp. were all found, to utilise ACC as the sole nitrogen source for growth. *Microbacterium* strain isolated from soil has been recently described to exhibit ACC deaminase activity, to produce siderophores, 1AA and to solubilize phosphates (Sheng *et al*., 2009).

Bacterial siderophores are an important class of compounds that enhance plant growth and protect the plant health by binding to available iron (Fe^{3+}) in soils. In the present study, all the rhizospheric and endophytic isolates are able to produce siderophores. Islam *et al*. (2009) found that 47.1% of the diazotrophic strains isolated from paddy fields were producing siderophore. However, these strains are restricted to *Serratia*, *Burkholderia* and *Herbaspirillum*. Shahi *et al*. (2011) reported 21 out of 114 isolates showed multiple growth promoting activity including production of siderophores. All of the diazotrophic *Burkholderia* sp. isolated from tomato are siderophore producers which could play a major role in the biocontrol of phytopathogens.

HCN production is a common trait within the group of *Pseudomonas* present in the rhizosphere. Some studies showed that about 50 % of pseudomonads isolated from potato and wheat rhizosphere were able to produce HCN *in vitro* (Schippers *et al*., 1990). Hydrogen cyanide production in *Pseudomonas* has also been reported by Siddiqui and Shakeel (2008). To date many different bacterial genera have shown to be capable of producing HCN including species of *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas* and *Rhizobium* (Ahmad *et al*., 2008). In the present study, most of the strains of *Bacillus*, *Klebsiella*, *Serratia* and *Enterobacter* sp. were found to produce HCN. The HCN production was not observed in *Enterobacter* sp. (BR1), *Stenotrophomonas* sp. (SS4), *K. pneumonia* (CR3), *Klebsiella* sp. (CB4), *Serratia* sp. (OR3), *Klebsiella* sp. (OR5), *Bacillus* sp.(CD2), *K. pneumonia* (SV1), *K. pneumonia* (CGE3), *Pantoea* sp. (PRE2) and *B. licheniformis* (CBE9). The absence of HCN production in these strains could indicate that this ability is not widely distributed among diverse free-living diazotrophic bacteria. Increase of plant growth includes a variety of mechanisms by which the bacteria prevent phytopathogens from inhibiting plant growth and development. This mechanism of plant growth promotion so far has been treated in a step motherly way as far as diazotrophs are

concerned. Results of the present study on antagonistic activity of diazotrophic isolates showed that, 4% of diazotrophic isolates are antagonistic to *X. oryzae*, 72% against *R. solani*, 68% against *S. oryzae* and 56% against *P. oryzae.* Jaiganesh *et al*. (2007) found that *S. marcescens* appeared to be an ideal agent for the control of *P. oryzae*, because it produced chitinolytic enzymes which cause degradation of the fungal cell walls, induction of plant defence reaction and certain antifungal low molecular weight molecules. Moreover, much evidence has indicated a potential role of such species in plant growth promotion properties as fungal biocontrol (Press *et al*., 1997). Many rhizospheric and endophytic bacteria are reported to have antagonistic activity against a variety of plant pathogens. Similar to our findings many strains of *Bacillus* have also been widely used as microbial inoculum for improving plant growth and/or for biocontrol of pathogens in trials with wheat, spinach, strawberry and tomato (Herman *et al*., 2008).

Phosphorus, potassium and zinc are important plant nutrients and the beneficial role of plant growth promoting bacteria in maintaining adequate levels of these mineral nutrients in crop production has been previously reported (Rodríguez and Fraga, 1999). It is observed that, all the diazotrophs are solubilize phosphorus, 28% of isolates could mineralize potassium and 44 % of isolates could solubilize zinc with varying ability. *Pantoea* sp. and *Bacillus* sp. have the P-solubilization capacity and could be used as inoculants and plant growth promoters to increase P-uptake by plants, as suggested by Park *et al*. (2005). Furthermore, the phosphate solubilization property and the presence of the nitrogen fixing genes in *S. marcescens* was demonstrated by Islam *et al.* (2009). A positive correlation between the potential for P and Zn solubilization has been reported (Wani *et al*., 2007). In the same way strains of *K. pneumoniae* (CR2), *Serratia* sp (CB2), *Serratia* sp (OR3), *S. marcescens* (CD1), *P. agglomerans* (ORE9), *Serratia* sp. (CRE9) and *Pseudomonas* sp.(CRE10) were able to solubilize all three tested minerals in the present investigation. Zinc solubilizing ability of *Bacillus* sp. and *Pseudomonas* sp. was assessed by Saravanan *et al*. (2003) using zinc oxide, zinc sulphide (sphalerite) and zinc carbonate in both plate and broth assays. ZSB O-1 (*Bacillus* sp.) recorded maximum dissolution of zinc sulphide.

5.6. Stress tolerance by diazotrophs

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH are major limiting factors in crop production because they affect almost all the functions of plants. In addition to the ability of a plant to modify its physiology and metabolism, including the synthesis of a range of defensive proteins, certain soil bacteria can also help the plants to mitigate the environmental stresses. The introduction and persistence ability of a strain are affected by a number of abiotic factors like high salt, high water potential, high pH and high temperature (Egamberdieva, 2009).

Soil salinity in arid regions is frequently an important limiting factor for cultivating agricultural crops. Although many technologies have been implicated in the improvement of salt tolerance, only PGPR-elicited plant tolerance against salt stress has been previously studied by Egamberdieva (2009). In the present study, 24% of isolates was able to tolerate a NaCl concentration of 7.5 %. Most of the strains having ability to produce ACC deaminase showed resistance to salt. From a point of view of salinity condition regulated in plant-associated rhizobacteria, some isolates including genera *Pseudomonas* and *Bacillus* have shown to have capacity to promote wheat growth in salinated soils of Uzbekistan (Egamberdieva *et al*., 2008). Similar results were reported in India in groundnut (*Arachis hypogaeae*) inoculated with ACC deaminase producing *Pseudomonas fluorescens*, according to Saravanakumar and Samiyappan (2007). In another study, the ethylene content in tomato seedlings exposed to high salt was reduced by application of *Achromobacter piechaudii*, indicating that bacterial ACC deaminase was functional. *A. piechaudii*, which produced ACC, increased the growth of tomato seedlings by as much as 66% in the presence of high salt contents (Mayak *et al*., 2004). In salt stressed conditions *K. pneumoniae* KHS2 grown with casamino acids as the nitrogen source, proline was the major osmolyte and both trehalose and glutamate were observed by Madkour *et al*. (1990). The availability of desiccation and salt tolerant PGPR appears to be advantageous especially in seasonally dry regions or regions with salinity problems, which is 40% of the world land surface (Cordovilla *et al*., 1994). In another work Chinnadurai *et al*. (2009) reported the presence of ACC deaminase activity among the phyllosphere methylobacteria of rice and

found that foliar spray of ACC deaminase positive methylobacterial isolates enhanced the root and shoot length of rice and tomato seedlings under gnotobiotic condition and lower the ethylene level (60–80%) in the plant species.

The test for temperature tolerance of 25 isolates from different grass species revealed that, 32% of isolates could grow at 55°C. The high temperature tolerance of 4 *Bacillus* isolates may be attributed to production of spores and heat shock proteins. The temperature tolerance of different strains of *Serratia* sp., *Klebsiella* sp. and *Pantoea* sp. may be due to high polysaccharide production during stress. Jenson (1981) reported N_2 fixing microorganisms survive during hot dry conditions upto 60ºC.

Survival of bacteria during desiccation is the result of many different factors. Desiccation tolerance is an indirect result of coping with stresses, such as osmotic, temperature, and oxygenic stresses. Despite the uncertainties in understanding desiccation responses of rhizobia, we know that desiccation conditions influence survival and that responses to a decrease in water activity and an increase in osmotic or salt stress elevate the ability of some microorganisms to survive desiccation. Microorganisms vary in their tolerance of decreasing water potential. Eventhough, water potential affects the activity of soil microorganisms. A direct relationship between starvation resistance and the ability of bacterial survival in soil was reported in earlier studies. All the selected diazotrophs are able survive under the permanent wilting point conditions (15 BAR) up to 90 days. Chen and Alexander (1997) related the growth of soil isolates at low water activity to their ability to survive desiccation. In disturbed soils in the laboratory, a minimum of 50% field capacity moisture was required for nitrogenase activity (Roper, 1985), whereas in *insitu* assays in undisturbed soils in the field, nitrogenase activity occurred at moistures below 30% field capacity (Roper, 1983). These results agree with the earlier findings, who also have reported survival of diazotrophs at low water potentials.

Present investigation, revealed the resistance of 24 diazotrophs against 5 heavy metals *viz*., Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} and it is observed that many of the isolates have multiple tolerance to heavy metals. It is generally observed that the firmicutes showed high resistance to heavy metals than others. In the same way, Dib *et al.* (2008)

reported that in some Gram positive bacteria other characteristics may help resistance, such as the spore forming ability in *Bacillus* and the relatively high GC content in Actinobacteria. Oliveira *et al.* (2009) attempted to isolate diazotrophic bacteria that were able to tolerate high concentrations of arsenic. In total, 22 arsenic resistant isolates showed ability to fix nitrogen. The finding showed that diazotrophic microbes (*Rhizobium* and *Azotobactor*) increased maize growth on heavy metal polluted soil, which might be due to reduction in metal toxicity on plant, because the growth on Pb added soil in the absence of these microbes was reduced significantly.

The toxicity is ranged from the highest toxic element (Hg^{+2}) to the lowest toxic element (Pb^{+2} and Al^{+3}). Low concentrations of certain heavy metals such as zinc are essential for many cellular processes of bacteria. However, higher concentrations of these metals often are cytotoxic. The microbial resistance to heavy metals is attributed to a variety of detoxifying mechanisms developed by resistant microorganisms, such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux, etc. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another. Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological and/or genetic as well as environmental modification of metal speciation (Wuertz and Mergeay, 1997). Microbes demonstrate various types of resistance mechanisms in response to heavy metals; these mechanisms may be encoded by chromosomal genes, but the most usual loci conferring resistance are located on plasmid. Alzubaidy (2012) reported that, growth rate of *S. marcescens* (S4) in the presence of heavy metals (Zn^{+2}, Fe^{+2}, Al^{+3}, Pb^{+2}) were consistently slower than that of the control.

The first two components (PC1 and PC2) of PCA were accounting for 49.7 % variability with PC1 contribution of 34.7 % and PC2 of 15.0 % (Fig.12).With reference to plant growth promoting traits of the diazotrophic isolates, ACC deaminase, growth hormone production, P, K and Zn releasing ability were positively ordinated (placed in the right handed top of the plot) with significant contribution to the PCs. The variables such as salt and temperature tolerance showed minor contribution to the PC loadings. When considering the isolates CR2 (*K. pneumonia),* OR3 (*Serratia* sp.), CRE9 (*Serratia* sp.) and ORE9 (*P. agglomerans*) were placed in the position of scoring

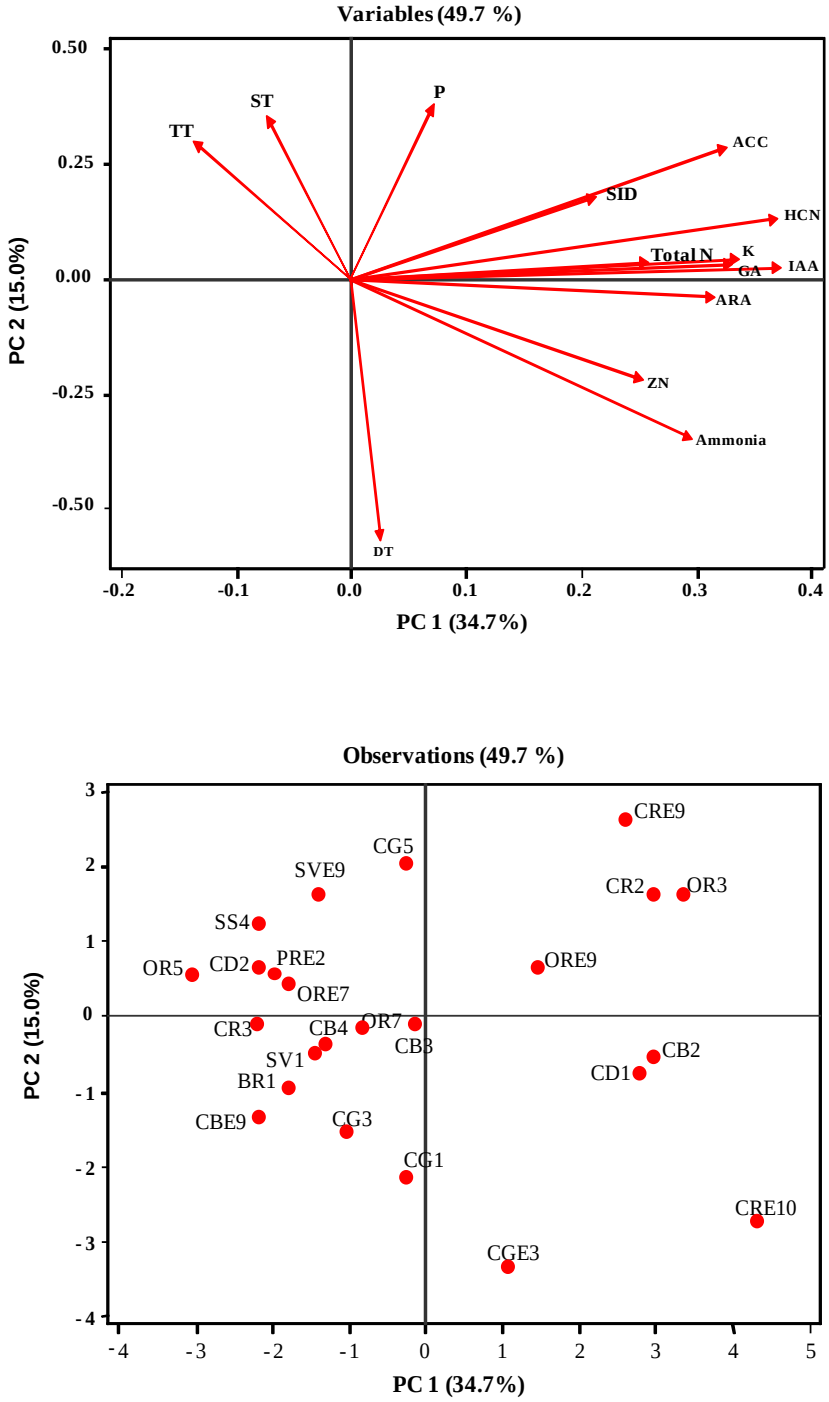

Fig. 12. Principal component analysis on screening of multiple growth promoting diazotrophs. A. Loading plot. B. Scoring plot. Total N- total Nitrogen content; Ammonia- ammonia excretion; ARA-Acetylene Reduction Assay; IAA-Indole Acetic Acid production; GA-Gibberellic acid production; ACC-ACC deaminase activity; SID-Siderophore production; HCN-Hydrogen cyanide (HCN) production; P-phosphorus solubilization; K-potassium releasing potential; Z-zinc solubilization; ST-Salt tolerance; TT-Temperature tolerance; DT-Dessication tolerance. *Klebsiella* sp. (CG1), *Enterobacter* sp. (CG3), *B.subtilis* (CG5), *Stenotrophomonas* sp. (SS4), *K. pneumoniae* (CR2), *Klebsiella* sp. (CB4), *K. pneumoniae* (CR3), *Serratia* sp.(CB2), *B. subtilis s* (CB3), *Serratia* sp. (OR3), *S. marcescens (*CD1), *K. pneumoniae* (SV1), *Klebsiella* sp. *(*OR5),*Klebsiella* sp. (OR7), *Bacillus* sp. (CD2), *Pseudomonas* sp.(CGE3), *Serratia* sp. (CRE9), *Pseudomonas* sp.*(*CRE10), *Enterobacter* sp.(BR1), *Bacillus* sp. (CBE9), *Enterobacter* sp (ORE7), *E. sacchari (*SVE9), *P. agglomerans* (ORE9), *Pantoea* sp.(PRE2).

plot, where most of the variables positioned in the loading plot. CBE9, BR1, CG3, SV1 like strains, having less multiple- functional plant growth promoting traits were ordinated in the negative plots. Some isolates like CG5, CB2, CD1 and CRE10 also considered as they have possible correlation with either one of the PCs.

5.7. Effect of diazotroph inoculation on the growth of rice under *in vitro*

Bioinoculants increase crop growth by a combination of mechanisms, which include biological nitrogen fixation (BNF), phytohormone production, increasing the availability of soil nutrients, and disease control (Cocking, 2003). In the present investigation, nitrogen fixing and phytohormone producing activities were found in many diazotrophic strains (*Klebsiella* sp.(CG1) *Enterobacter* sp.(CG3), *Bacillus* sp. (CG5), *K. pneumoniae* (CR2), *K. pneumoniae* (CR3), *Serratia* sp.(CB2), *B. subtilis* (CB3), *S. marcescens* (CD1) *K. pneumonia* (SV1), *Serratia* sp.(OR3) *Pseudomonas* sp. (CGE3), *Serratia* sp. (CRE9), *Pseudomonas* sp. (CRE10), *Enterobacter* sp.(ORE7) and *P. agglomerans* (ORE9). Moreover, the other plant growth promotion properties such as mineral solubilizing and biocontrol properties, *nif H* gene amplification of isolates have been well documented. It is therefore probable that they can be used as bacterial inocula to support growth and development of rice plants *in vitro* and in field experiment.

In the present study, gnotobiotic assay inoculation effects of diazotrophic bacteria on rice were studied. It clearly revealed that diazotrophs aided in production of healthy and vigorous rice seedlings. All of the strains selected for gnotobiotic study showed significant nitrogenase activity, and amplification of the *nifH* gene confirmed their ability to fix nitrogen. The dehulled and surface sterilized seeds treated with diverse diazotrophic strains showed a 59% and 66% increase in root length as well as shoot length respectively when compared with control. Islam *et al*. (2009) reported that inoculation of rice with free-living diazotrophic bacteria remarkably increased plant height and dry biomass production compared with the control under greenhouse conditions. George *et al*. (2013) found that significant increase in growth and nutrient uptake accompanied with higher populations of plant beneficial microorganisms in their rhizospheres were recorded up on inoculation with *Serratia* sp. and *Klebsiella* sp.

5.8. Endophytic colonization of diazotrophic endophytes *in vitro*

In the present study, scanning electron microscopy analysis confirmed the presence of endophytic bacteria *viz*., *Serratia* sp. (CRE9), *Pseudomonas* sp. (CRE10), *Pseudomonas* sp. (CGE3) and *P. agglomerans* (ORE9) were able to colonize in great extent the root surface and inner part of the rice plants. According to our results, endophytic bacteria seem to be uniformly distributed on the rhizoplane of the root and also we identified colonization at the intercellular junction. Electronmicroscopic studies of *A. diazotrophicus* after infection of sugarcane tissues clearly provided detailed information about the localization of *A. diazotrophicus* cells within sugarcane tissues of microprogagated plantlets (James *et al*., 1994).

This finding is probably due to the fact that intercellular regions represent more space and opportunity for the movement of endophytes; besides, very probably the mucilaginous layer, which covers the epidermis of the root, has a lower tension in these regions in accordance with Bowen (1979). The close association between a plant and an endophyte may provide suitable conditions for nutrient transfer between the bacteria and their host, than the association between predominantly rhizosphere bacteria and plants (Stoltzfus and de Bruijn, 2000). Previous reports indicate that, at the intercellular regions, there is increase in the concentration of carbon as a source of energy, thus explaining the preference of bacteria for this part of the root (Bennett and Lynch, 1981). *Azoarcus* strain BH72 is able to invade rice roots and colonize the cortex cells of roots as well as the stem bases and the shoot (Hurek *et al*., 1994). The systemic spreading of *Azoarcus* in the plant is facilitated by cellulolytic activities (Reinhold-Hurek *et al*., 1993).

It has been suggested that bacteria could develop on the surface of the epidermal cells and on the cellular junctions. Inoculation of the endophytic bacteria *Pseudomonas* sp. (CRE 10) recorded maximum shoot length and root length (64% and 65% respectively). Results indicate the presence of many filaments cross linking the endophytic bacteria and with the rhizoplane, suggesting a structural compatibility between endophytes and the cell wall. Diazotrophs specifically interacts with rice roots through a sequence of three neutral sugars and glucuronic acid; this interaction allows for an efficient colonization. We identified high densities of endophytic bacteria in emerging zones from the lateral

roots and particularly, in the basal parts. This finding agrees with other studies indicating that these parts of the roots are highly susceptible to disruption, causing the release of endophytes (Agarwhal and Shende, 1987). The entry of diazotrophic grass endophytes *Azoarcus* sp. BH72 into the root is most likely an active process, which might be mediated by enzymes degrading plant cell wall polymers (Reinhold-Hurek *et al*., 1993). In contrast to most phytopathogens harboring plant cell wall-degrading enzymes, the products are not metabolized by *Azoarcus* sp. BH72, they grow neither on cellulose, cellobiose or glucose or any other carbohydrate. Moreover, the enzymes are unlike in pathogens not efficiently excreted into the culture supernatant, but remain bound to the cell surface (Reinhold-Hurek *et al*., 1993). Once bacterial cells have crossed the exodermal barrier, they can remain at the site of entry as it has been shown for *Paenibacillus polymyxa* in *Arabidopsis* (Timmusk *et al*., 2005) or move deeper inside and occupy the intercellular space of the cortex (James *et al*., 1994; Roncato-Maccari *et al*., 2003; Compant *et al*., 2005 and Gasser *et al*., 2011). This might cause a less aggressive attack of plant cells by an endophyte in comparison to plant pathogens. The preferable sites of bacterial attachment and subsequent entry are the apical root zone with the thin-walled surface root layer such as the cell elongation and the root hair zone (zone of active penetration), and the basal root zone with small cracks caused by the emergence of lateral roots (zone of passive penetration). At these sites bacteria are often arranged in micro-colonies comprising several hundreds of cells (Zachow *et al*., 2010). The exact mechanism used by the endophytic bacteria to penetrate and colonize the endorhizosphere of the rice remains unclear, and more studies are needed to understand better the mechanism of penetration as well as the exact role of endophytic bacteria.

5.9. Influence of diazotrophic bacterial inoculation on growth and yield of rice under lowland ecosystem

In the present investigation, selected diazotrophs were found to produce phytohormones such as IAA, Gibberellic acid (GA3), produce siderophores that can provide iron to plants, solubilize minerals such as P, K, Zn and synthesize enzymes such as ACC deaminase that can modulate plant growth and development. Inoculation of plants with these diazotrophs increases plant growth under gnotobiotic conditions. Since the selected diazotrophs were used as bacterial inocula to evaluate the growth and development of rice plants in the field conditions.

In the present study, it was observed that inoculation with diazotroph resulted in an increase in plant height. *Serratia* sp. (CB2), *Klebsiella* sp. (CG1), *Bacillus* sp .(CG5), *K. pnemoniae* (CR2) and *Pseudomonas* sp.(CRE10) increased the rice plant height 2.7% - 9.1% through nitrogen fixation and phytohormone production as reported by Keyeo *et al*. (2011). Tillering is an important phenological event in rice development. Tran Van *et al*. (2000) noticed an increase in number of effective tillers when *Burkholderia vietnamiensis* was inoculated to rice. Inoculation of *S. marcescens* (CD1), *Serratia* sp. (CB2) and *K. pnemoniae* (CR2) increased the yield by 5.9% - 32%. This increase in yield could be the result of production of growth hormones, solubilization of minerals and biological N_2 fixation and biocontrol activity of diazotrophic inoculants. Das and Saha (2003) observed increases of up to 20% in grain yield in rice in response to inoculation with *Azotobacter* spp. and *Azospirillum* spp., but these increases were less than those recorded with optimum N fertilizer application. Baldani *et al*. (1987) found increases in grain yield in wheat up to 31% following inoculation with *Azospirillum* spp., but due to variability in the trials, there were no statistical differences between inoculated plants and untreated controls.

Verma *et al*. (2001) mentioned that most of the soils under rice cultivation contain insoluble phosphates. Therefore, the ability of rhizobacteria to solubilize precipitated phosphates and enhances phosphate availability to rice, represents a possible mechanism of plant growth promotion under field conditions. In our study the *S. marcescens* (CD1) showed maximum solubilization of phosphorus that might have helped the crop growth. Similar to our results, George *et al*. (2013) mentioned that *S. marcescens* exhibited N- fixation potential, phosphate solubilization, ammonification, and production of indole acetic acid, 1-aminocyclopropane-1-carboxylate-deaminase activity, chitinase activity, siderophore production and antibiotics. In addition, seed bacterization with *S. marcescens* (CD1) increased the growth parameters of test plants such as paddy and cowpea over uninoculated control in green house assay. Biswas *et al*. (2000) reported the growth promoting effect of diazotrophic bacterial strains of *Herbaspirillum seropedicae*, *Glucanacetobacter diazotrophicus*, *Azospirillum brasilense*, *Burkholderia cenocepacia* on rice. Okon and Labandera-Gonzalez (1994), reported, 3–32% increase in grain yield from 70% diazotrophs of the inoculated trials. Such results clearly demonstrate the plant growth promotion in rice by bioinoculants. Yim *et al*. (2009) reported, inoculation of the

diazotrophic bacterial strains significantly increased the biomass of plants and also 37% increase on the total N content in plant tissues when compared to uninoculated control as found in our study. Similarly De broy *et al*. (2009) showed that seed inoculation of diazotrophs on rice have improved growth, nitrogen content, grain weight and yield of crops. Inoculation of *K. pnemoniae* (CR2) and *Serratia* sp.(CB2) strain stimulated the plant growth, the grain yield and the accumulation of potassium and magnesium in the wheat grain (Ruppel, 1989). Our findings indicate that most of the selected strains possess multiple plant growth promoting properties that significantly improve the growth of the rice when tested under field conditions. In the "additive hypothesis", it was suggested that multiple mechanisms, such as dinitrogen fixation, phosphate solubilization, and ACC deaminase activity, together with IAA biosynthesis, are responsible for the observed plant growth promotion and yield increase (Bashan and Holguin, 1997). Inoculation of plants with plant growth promoting bacteria not only increases plant growth but also improves total NPK uptake (Shaharoona *et al*., 2007 and Wu *et al*., 2005). Biological nitrogen fixation (BNF) technology can play an important role in substituting the use of chemical N fertilizers in rice cultivation.

Principal component analysis clearly correlate the observed variables and bioinoculants tested under field condition and showed that 64.3% variability with PC1 contribution of 46.9% and PC2 of 17.3 % (Fig.13). Principal component analysis of variables showed two quartet among variables while number of tillers, panicle length, effective tillers, nitrogen uptake, arginine deaminase activity, straw yield, grain yield, test weight and plant height are 46.9% contributed to isolates performance. In case of gnotobiotic experiment variables (17.3%) such as dry matter production, root length and shoot length are not much influenced by diazotrophic isolates performance. Treatment number T4 (*Serratia* sp.) and T5 (*K. pneumoniae*) have the positive score value while treatments such as T6, T3, T1 and T2 are closer to positive side. The results indicated that T4 (*Serratia* sp.) and T5 (*K. pneumoniae*) inoculated plots strongly influence the productive tiller counts, nitrogen uptake, grain yield and enzyme activity. These results are also in conformity with the correlation analyses (Table 37).

The novel efficient isolates of *Klebsiella pneumoniae* and *Serratia* sp. with multifaceted plant growth promoting activity obtained from the weedy grass rhizosphere under stressed condition may be employed in nutrient deficient and problematic soils for stress mitigation and sustainable crop cultivation with fewer chemical inputs.

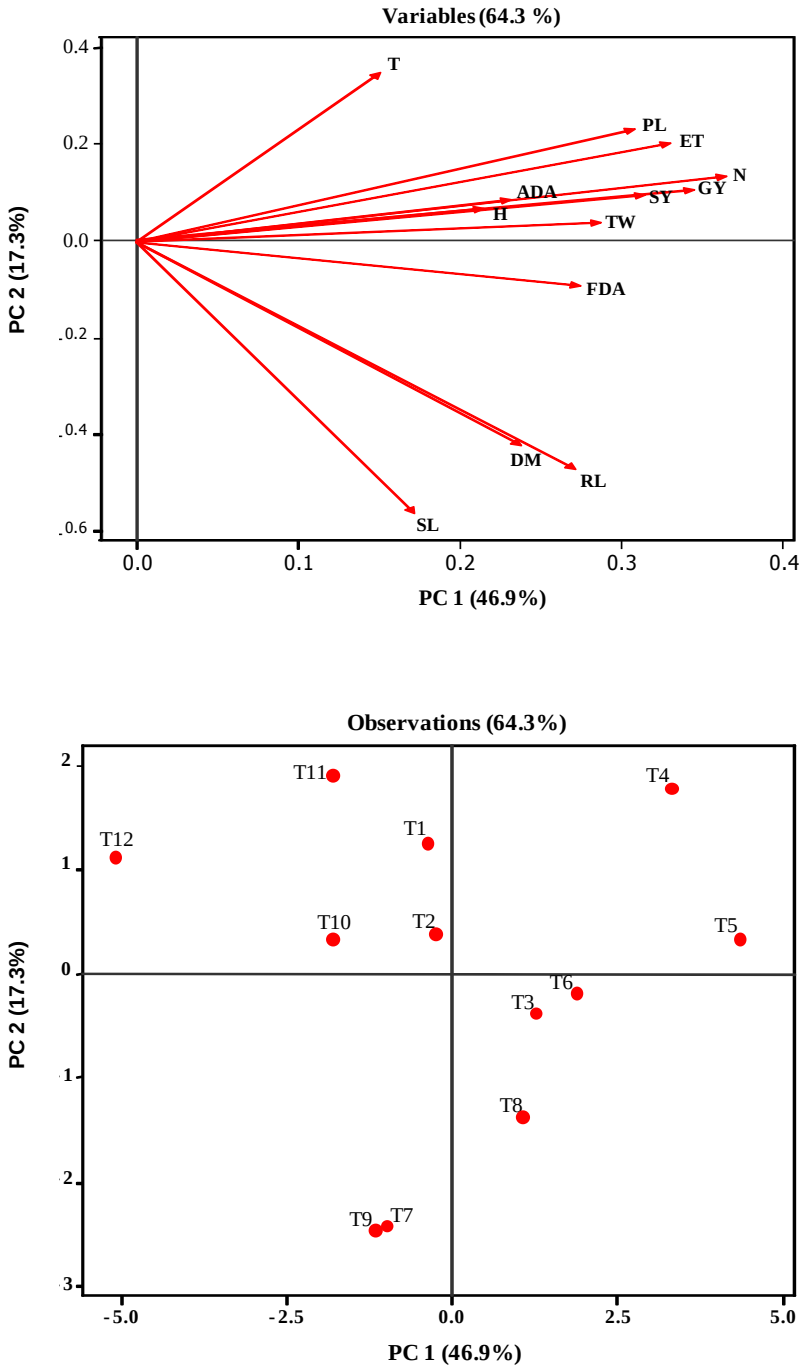

Fig. 13. Principal component analysis on influence of diazotrpophs inoculation on growth of rice under gnotobiotic and field conditions. A. Loading plot; B. Scoring plot; T– Number of tillers; Pl- Panicle length; ET- Effective tillers; ADA-arginine deaminase activity; H-Height; N- Nitrogen uptake; SY-straw yield; GY -grain yield; TW- test weight; FDA- fluorescein diacetate hydrolysis ; DM- Dry matter production; RL –root length ; SL- Shoot length; T1-*Klebsiella* sp.(CG1)+75% RDF; T2-*B.subtilis* (CG5)+75% RDF ; T3- *S. marcescens* (CD1)+75% RDF; T4- *Serratia* sp. (CB2)+75% RDF; T5- *K. pneumonia* (CR2)+75% RDF ;T6- *Serratia* sp.(OR3)+75% RDF; T7- *Serratia* sp. (CRE9)+75% RDF; T8- *Pseudomonas* sp.(CRE10)+75% RDF; T9- *P. agglomerans* (ORE9)+75% RDFT; 10- *A.lipoferum* (Az 204)+75%RDF;T11-100% RDF;T12-Control (Absolute control)

Summary

CHAPTER VI

SUMMARY

In the present investigation, diazotrophic bacteria were isolated from rhizosphere and internal tissues of ten selected grass species. The diazotrophic bacteria were screened and identified using 16S rDNA sequencing. The presence of *nif* gene was detected by PCR using universal primers. These isolates were found to produce multiple plant growth promoting substances and showed tolerance to various abiotic stress conditions. Gnotobiotic experiment was conducted to study the influence of efficient diazotrophic isolates on the growth of rice. Further, the efficient cultures were evaluated for their performance in rice under field conditions. The results obtained in the experiments are summarized as follows:

1. Based on their availability in each physiographic region, a total of 10 different grass species (*Brachiaria reptans, Cenchrus glaucus, Saccharum spontaneum, Panicum repens, Cyperus rotundus, Dactyloctenium aegyptium, Chloris barbata, Oryza rufipogon, Cyanodon dactylon* and *Setaria verticillata*) along with the rhizosphere soils were sampled.

2. The physio-chemical and biological properties of samples were analysed and showed significant difference among the regions.

3. A total of 60 diazotrophic isolates from rhizosphere and twenty one putative endophytic isolates were obtained from all the 10 grasses. A total of 30 rhizospheric and 20 entophytic diazotrophic isolates were selected based on the growth on N- free medium, total nitrogen content and ammonia excretion.

4. As many as 60 diazotrophic isolates from rhizosphere and 21 putative endophytes were obtained by using four different N-free media. These isolates were further analyzed for growth on N- free medium, total nitrogen content and ammonia production.

5. Among the isolates, the highest nitrogenase activity was exhibited by isolate OR3 (172.25 ±13.95 n moles of ethylene mg^{-1} of protein h^{-1}) isolated from *Oryza rufipogon.* Among the endophytic diazotrophs, the highest ARA activity was

found in CGE3 (171.45 ±11.80 n moles of ethylene mg^{-1} of protein h^{-1}) isolated from *Cenchrus glaucus*.

6. The selected both rhizosphere and endophytic diazotrophic bacterial colonies were characterized by cell shape, morphology and Gram reaction.
7. The intrinsic antibiotic resistance and carbon-substrate utilization profiling of the selected isolates showed remarkable differences among them, forming seven different clusters.
8. The 16S rRNA gene sequence homology revealed the presence of diversity of γ Proteobacteria and Firmicutes. The authenticated diazotrophic isolates of grass species were screened for the presence of *nifH* gene and the amplification could be clearly detected in 15 isolates.
9. Plant growth promoting traits of all the diazotrophs isolates from grass species were analysed. Among the rhizosphere diazotrophic isolates, the amount of IAA and GA production, P-solubilization, siderophore and HCN production were higher in *Serratia marcescens* (CD1).
10. Among the endophytic diazotrophic isolates, *Pseudomonas* sp. (CRE10) showed maximum multiple- plant growth promoting traits. Seven rhizospheric and five endophytic isolates produced ACC deaminase and thereby regulated the ethylene concentration.
11. Most of the selected diazotrophic isolates showed antagonistic activity against sheath blight (*Rhizoctonia solani)*, blast (*Pyricularia oryzae*) and sheath rot (*Sarocladium oryzae*) of rice.
12. Tolerance level to various abiotic stresses such as salt, temperature, dessication and heavy metals were assessed and the results revealed that these rhizosphere isolates *viz*., *Bacillus* sp. (CG5), *Stenotrophomonas* sp. (SS4) and *Bacillus* sp. (CD2) and the endophytic isolates *viz*., *E. sacchari* (SVE9), *Bacillus* sp. (CBE9) and *Serratia* sp. (CRE9) registered their remarkable ability to tolerate salt upto 7.5%.
13. The temperature level (55ºC) had profound influence on the population of diazotrophs and moisture potentials (field capacity and permanent wilting point) were not found

to significantly influence the population of diazotrophs. The diazotrophic isolates *Bacillus* sp. (CG5), *Bacillus* sp. (CBE9) and *E. sacchari* (SVE9) showed maximum resistance to heavy metals such as Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}.

14. Under gnotobiotic conditions, *Pseudomonas* sp. (CRE10) inoculation registered maximum shoot length and root length in rice. Plant dry matter production was higher in *S. marcescens* (CD1) than the other strains.

15. The endophytic colonization in the rice seedlings (roots and culm) were examined by SEM analysis. Results indicated that endophytic bacteria were preferentially colonized in the rhizoplane and inner side of rice roots.

16. A field experiment was carried out to evaluate the efficiency of elite strains on the growth and yield of rice (cultivar- ADT 43) under lowland ecosystem. Among the diazotrophic strains, *Serratia* sp. (CB2) isolated from *Chloris barbata* enhanced plant growth, productive tiller counts, and nitrogen uptake and enzyme activity.

17. Among the strains, *K. pneumoniae* (CR2) and *Serratia* sp. (CB2) registered grain yield increase of 32 and 31% respectively compared to the 100% NPK.

The present compilation of multiple plant growth promoting traits of diverse diazotrophs suggests that these organisms can promote plant growth by more than one mechanism and hence these traits could be better exploited. Besides exploring the potential for biological nitrogen fixation and other promising plant growth promoting functions carried out by diazotrophs, it is also important to ensure that the bacteria are well adapted to environmental conditions before they are utilized as inoculant strains. These diazotrophs could be very useful in the formulation of new microbial inocula and could be applied most profitably to economically important non-legume crops. In conclusion, this study demonstrates the occurrence of diverse groups of plant growth promoting diazotrophs in grass species. These beneficial microbes can be utilized in improving plant growth and yield in rice.

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Appendices

ANNEXURE-1

Table 1. Effect of salt concentration on the growth of diazotrophs isolated from rhizosphere of grass species

Isolate	Population (log cfu ml^{-1}) at different level of salt concentration (NaCl%)																			
	1%					2.5%					5%					7.5%				
	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h
Enterobacter sp.(BR1)	4.1 (±0.7)	5.4 (± 0.6)	6.5 (± 0.4)	8.5 (± 0.3)	9.3 (± 0.3)	3.3 (± 0.2)	4.5 (± 0.2)	5.4 (± 0.2)	5.6 (± 0.9)	6.7 (± 0.3)	3.1 (± 0.6)	4.3 (± 0.2)	5.1 (± 0.1)	4.1 (± 0.3)	3.3 (± 0.7)	ND	ND	ND	ND	ND
Klebsiella sp.(CG1)	4.1 (± 0.4)	5.7 (± 0.1)	6.5 (± 0.4)	7.5 (± 0.1)	8.2 (± 0.3)	4.3 (± 0.1)	5.5 (± 0.1)	5.7 (± 0.4)	6.8 (± 0.6)	6.6 (± 0.7)	4.1 (± 0.2)	5.1 (± 0.2)	5.1 (± 0.6)	4.1 (± 0.3)	3.3 (± 0.7)	ND	ND	ND	ND	ND
Enterobacter sp.(CG3)	3.4 (± 0.4)	5.4 (± 0.6)	5.6 (± 0.6)	6.2 (± 0.7)	8.2 (± 0.4)	4.3 (± 0.2)	5.5 (± 0.2)	5.1 (± 0.4)	4.1 (± 0.1)	4.3 (± 0.3)	3.2 (± 0.2)	5.0 (± 0.2)	5.2 (± 0.3)	4.1 (± 0.6)	2.2 (± 0.8)	ND	ND	ND	ND	ND
Bacillus sp. (CG5)	4.1 (± 0.3)	5.4 (± 0.8)	5.6 (± 0.4)	7.3 (± 0.7)	8.3 (± 0.2)	3.2 (± 0.4)	5.4 (± 0.1)	6.3 (± 0.2)	7.4 (± 0.3)	7.7 (± 0.3)	3.3 (± 0.5)	5.2 (± 0.3)	6.1 (± 0.4)	5.3 (± 0.1)	4.3 (± 0.5)	4.3 (± 0.1)	4.3 (± 0.1)	4.1 (± 0.1)	2.7 (± 0.1)	2.4 (± 0.1)
Stenotrophomonas sp. (SS4)	3.4 (± 0.1)	5.6 (± 0.1)	6.0 (± 0.6)	6.1 (± 0.4)	8.3 (± 0.3)	3.2 (± 0.1)	4.3 (± 0.1)	5.1 (± 0.1)	5.3 (± 0.1)	7.1 (± 0.2)	3.1 (± 0.3)	4.3 (± 0.3)	5.3 (± 0.4)	5.4 (± 0.1)	6.7 (± 0.7)	2.5 (± 0.5)	3.8 (± 0.3)	2.5 (± 0.1)	1.7 (± 0.1)	1.4 (± 0.1)
K. pneumoniae(CR2)	4.1 (± 0.1)	5.5 (± 0.1)	6.1 (± 0.5)	6.9 (± 0.2)	7.9 (± 0.5)	4.1 (± 0.5)	5.4 (± 0.5)	5.9 (± 0.1)	5.9 (± 0.4)	6.9 (± 0.3)	3.1 (± 0.3)	4.2 (± 0.2)	5.4 (± 0.1)	5.5 (± 0.2)	4.4 (± 0.5)	ND	ND	ND	ND	ND
K. pneumoniae (CR3)	4.1 (± 0.2)	5.5 (± 0.2)	6.2 (± 0.1)	7.1 (± 0.1)	8.2 (± 0.1)	3.3 (± 0.5)	4.3 (± 0.5)	5.2 (± 0.2)	5.3 (± 0.2)	5.6 (± 0.2)	4.1 (± 0.4)	5.2 (± 0.6)	6.5 (± 0.2)	6.5 (± 0.8)	3.3 (± 0.9)	ND	ND	ND	ND	ND
Serratia sp. (CB2)	4.3 (± 0.4)	5.4 (± 0.5)	6.4 (± 0.6)	7.4 (± 0.8)	8.4 (± 0.7)	3.3 (± 0.6)	5.3 (± 0.3)	6.0 (± 0.5)	6.3 (± 0.6)	6.5 (± 0.7)	3.2 (± 0.3)	4.2 (± 0.3)	5.7 (± 0.4)	3.5 (± 0.7)	2.5 (± 0.8)	ND	ND	ND	ND	ND
B. subtilis (CB3)	4.3 (± 0.1)	5.3 (± 0.1)	6.2 (± 0.1)	7.2 (± 0.2)	8.0 (± 0.2)	3.1 (± 0.2)	5.2 (± 0.1)	6.3 (± 0.7)	6.3 (± 0.5)	6.3 (± 0.1)	4.3 (± 0.1)	5.3 (± 0.4)	5.4 (± 0.3)	4.6 (± 0.7)	4.8 (± 0.2)	2.6 (± 0.1)	3.7 (± 0.1)	2.5 (± 0.1)	2.5 (± 0.1)	1.9 (± 0.1)
Klebsiella sp.(CB4)	4.3 (± 0.3)	5.6 (± 0.3)	6.2 (± 0.6)	7.1 (± 0.6)	8.4 (± 0.8)	3.3 (± 0.1)	4.2 (± 0.7)	5.2 (± 0.4)	5.3 (± 0.1)	5.3 (± 0.1)	3.2 (± 0.1)	4.4 (± 0.1)	5.4 (± 0.8)	3.4 (± 0.1)	2.5 (± 0.3)	ND	ND	ND	ND	ND
Serratia sp.(OR3)	3.3 (± 0.6)	5.4 (± 0.1)	6.3 (± 0.6)	7.3 (± 0.5)	8.3 (± 0.1)	3.3 (± 0.1)	5.3 (± 0.1)	6.0 (± 0.2)	6.1 (± 0.1)	6.4 (± 0.4)	3.3 (± 0.3)	4.4 (± 0.2)	5.1 (± 0.7)	4.4 (± 0.7)	3.3 (± 0.8)	ND	ND	ND	ND	ND
S. saprophyticus (OR5)	3.3 (± 0.1)	5.6 (± 0.1)	7.2 (± 0.2)	8.3 (± 0.1)	9.1 (± 0.1)	4.3 (± 0.5)	5.2 (± 0.5)	5.9 (± 0.6)	5.4 (± 0.7)	6.3 (± 0.8)	3.2 (± 0.5)	4.5 (± 0.4)	4.3 (± 0.6)	3.1 (± 0.4)	2.4 (± 0.7)	ND	ND	ND	ND	ND
Klebsiella sp.(OR7)	4.2 (± 0.5)	5.5 (± 0.1)	5.9 (± 0.1)	6.6 (± 0.3)	8.1 (± 0.4)	4.3 (± 0.3)	5.3 (± 0.1)	5.1 (± 0.1)	5.7 (± 0.1)	6.6 (± 0.1)	3.2 (± 0.4)	4.5 (± 0.3)	5.2 (± 0.8)	3.4 (± 0.2)	3.2 (± 0.5)	ND	ND	ND	ND	ND
S. marcescens (CD1)	4.2 (± 0.2)	5.3 (± 0.4)	6.1 (± 0.5)	7.1 (± 0.1)	8.2 (± 0.1)	5.2 (± 0.7)	5.5 (± 0.5)	6.2 (± 0.1)	7.1 (± 0.2)	7.3 (± 0.2)	3.2 (± 0.2)	5.2 (± 0.3)	5.1 (± 0.2)	3.5 (± 0.2)	2.2 (± 0.7)	ND	ND	ND	ND	ND
Bacillus sp.(CD2)	3.3 (± 0.1)	5.3 (± 0.1)	6.3 (± 0.3)	6.3 (± 0.2)	8.2 (± 0.2)	3.3 (± 0.1)	4.4 (± 0.3)	4.7 (± 0.3)	6.0 (± 0.2)	6.3 (± 0.4)	3.3 (± 0.5)	3.1 (± 0.2)	5.6 (± 0.2)	3.4 (± 0.5)	3.5 (± 0.9)	ND	ND	ND	ND	ND
K. pneumoniae (SV1)	4.3 (± 0.4)	5.3 (± 0.1)	6.3 (± 0.6)	7.3 (± 0.1)	8.1 (± 0.1)	3.3 (± 0.2)	5.1 (± 0.1)	6.3 (± 0.2)	6.3 (± 0.1)	7.3 (± 0.1)	3.2 (± 0.1)	5.2 (± 0.1)	4.7 (± 0.8)	4.6 (± 0.2)	3.5 (± 0.1)	ND	ND	ND	ND	ND
A.lipoferum (Az 204)**	4.1 (± 0.2)	5.4 (± 0.1)	5.6 (± 0.2)	6.3 (± 0.1)	8.4 (± 0.1)	3.4 (± 0.1)	4.4 (± 0.2)	5.5 (± 0.1)	6.5 (± 0.1)	7.5 (± 0.1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are mean (± SE) (n=3); *hours after inoculation; **standard culture; ND-not detected

Table 2. Effect of salt concentration on the growth of diazotrophs isolated from the internal tissues of grass species

Isolate	Population (log cfu ml^{-1}) at different lvel of salt concentration (NaCl%)																			
	1%					2.5%					5%					7.5%				
	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h
Pseudomonas sp.(CGE3)	4.1 (± 0.1)	4.9 (± 0.3)	7.1 (± 0.4)	9. 1 (± 0.1)	10.5 (± 0.1)	4.0 (± 0.4)	5.6 (± 0.5)	6.9 (± 0.4)	6.1 (± 0.4)	7.3 (± 0.2)	2.5 (± 0.1)	4.3 (± 0.1)	5.0 (± 0.1)	4.1 (± 0.1)	2.9 (± 0.2)	ND	ND	ND	ND	ND
Pantoea sp.(PRE2)	4.2 (± 0.2)	5.5 (± 0.2)	6.1 (± 0.5)	6.4 (± 0.1)	8.0 (± 0.5)	4.9 (± 0.3)	5.4 (± 0.6)	6.1 (± 0.5)	7.6 (± 0.2)	8.1 (± 0.2)	4.1 (± 0.1)	5.1 (± 0.1)	5.5 (± 0.6)	4.1 (± 0.6)	2.9 (± 0.4)	ND	ND	ND	ND	ND
Bacillus sp.(CRE9)	4.1 (± 0.5)	5.1 (± 0.6)	5.3 (± 0.5)	7.1 (± 0.5)	8.0 (± 0.2)	3. 5 (± 0.6)	5.3 (± 0.8)	6.2 (± 0.9)	7.9 (± 0.8)	8.4 (± 0.6)	3.3 (± 0.4)	5.1 (± 0.3)	6.1 (± 0.3)	5.3 (± 0.3)	4.4 (± 0.4)	4.3 (± 0.1)	4.3 (± 0.1)	4.1 (± 0.1)	2.3 (± 0.1)	2.7 (± 0.5)
Klebsiella sp.(CRE10)	2.9 (± 0.6)	4.8 (± 0.4)	6.6 (± 0.6)	7.6 (± 0.5)	8.7 (± 0.5)	4.1 (± 0.4)	5.4 (± 0.5)	3.4 (± 0.3	5.9 (± 0.5)	6.3 (± 0.3)	3.2 (± 0.5)	5.0 (± 0.3)	5.2 (± 0.3)	4.1 (± 0.9)	3.1 (± 0.1)	ND	ND	ND	ND	ND
B.licheniformis(CBE9)	4.2 (± 0.5)	5.3 (± 0.7)	6.6 (± 0.6)	7.4 (± 0.5)	9.0 (± 0.2)	3.0 (± 0.4)	4.1 (± 0.4)	5.0 (± 0.3)	5.0 (± 0.2)	6.4 (± 0.3)	4.0 (± 0.3)	5.1 (± 0.2)	6.2 (± 0.1)	6.2 (± 0.2)	3.2 (± 0.1)	2.5 (± 0.1)	3.8 (± 0.1)	2.5 (± 0.3)	1.6 (± 0.1)	1.3 (± 0.4)
Enterobacter sp.(ORE7)	3.2 (± 0.4)	4.7 (± 0.5)	5.7 (± 0.5)	6.3 (± 0.5)	8.1 (± 0.8)	3.5 (± 0.5)	4.5 (± 0.4)	5.2 (± 0.7)	5.1 (± 0.3)	7.1 (± 0.2)	3.0 (± 0.1)	4.0 (± 0.2)	5.1 (± 0.4)	5.1 (± 0.3)	6.5 (± 0.1)	ND	ND	ND	ND	ND
P .agglomerans (ORE9)	4.4 (± 0.1)	5.7 (± 0.4)	5.0 (± 0.6)	7.0 (± 0.6)	7.6 (± 0.2)	3.6 (± 0.3)	4.9 (± 0.3)	5.7 (± 0.5)	5.8 (± 0.5)	6.9 (± 0.3)	3.1 (± 0.3)	4.3 (± 0.4)	5.0 (± 0.6)	3.1 (± 0.6)	2.3 (± 0.2)		ND	ND	ND	ND
Bacillus sp.(SVE9)	4.0 (± 0.4)	5.2 (± 0.2)	6.2 (± 0.5)	7.2 (± 0.6)	8.7 (± 0.5)	3.1 (± 0.5)	5.9 (± 0.5)	6.1 (± 0.4)	6.3 (± 0.2)	6.8 (± 0.4)	3.1 (± 0.1)	4.2 (± 0.1)	5.0 (± 0.5)	4.1 (± 0.1)	4.2 (± 0.4)	2.5 (± 0.4)	3.7 (± 0.3)	2.5 (± 0.4)	2.5 (± 0.3)	1.9 (± 0.2)
** *G. diazotropicus* (PAL5)	3.3 (± 0.1)	5.3 (± 0.2)	6.3 (± 0.1)	7. 2 (± 0.2)	7.4 (± 0.8)	3.6 (± 0.5)	5.4 (± 0.4)	6.8 (± 0.1)	6.7 (± 0.4)	7.1 (± 0.4)	3.2 (± 0.3)	4.3 (± 0.2)	5.1 (± 0.7)	4.3 (± 0.5)	3.3 (± 0.1)	ND	ND	ND	ND	ND

Values are mean (± SE) (n=3); *hours after inoculation; **standard culture; ND-not detected

Table 3. Effect of temperature on the survival of diazotrophs isolated from the rhizosphere of different grass species

Isolate	Population (log cfu ml^{-1}) at different level of temperature																			
	40ºC					45ºC					50ºC					55ºC				
	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h
Enterobacter sp.(BR1)	3.3 (± 0.1)	5.2 (± 0.1)	6.1 (± 0.1)	8.4 (± 0.1)	9.4 (± 0.2)	4.2 (± 0.4)	5.5 (± 0.5)	5.4 (± 0.1)	6.1 (± 0.3)	6.5 (± 0.2)	3.1 (± 0.4)	4.3 (± 0.2)	5.0 (± 0.1)	4.1 (± 0.2)	3.0 (± 0.1)	ND	ND	ND	ND	ND
Klebsiella sp.(CG1)	3.2 (± 0.1)	5.2 (± 0.1)	5.4 (± 0.1)	7.4 (± 0.1)	9.4 (± 0.1)	4.3 (± 0.1)	5.5 (± 0.2)	3.1 (± 0.3)	5.0 (± 0.3)	6.3 (± 0.5)	3.2 (± 0.7)	5.5 (± 0.1)	5.2 (± 0.7)	4.1 (± 0.8)	3.1 (± 0.5)	ND	ND	ND	ND	ND
Enterobacter sp.(CG3)	4.1 (± 0.2)	5.4 (± 0.3)	6.3 (± 0.4)	7.2 (± 0.3	9.4 (± 0.5)	4.0 (± 0.1)	5.3 (± 0.1)	5.8 (± 0.1)	5.9 (± 0.3)	6.8 (± 0.2)	3.0 (± 0.6)	5.2 (± 0.4)	5.3 (± 0.7)	5.5 (± 0.1)	5.3 (± 0.5)	ND	ND	ND	ND	ND
Bacillus sp. (CG5)	4.0 (± 0.1)	5.4 (± 0.1)	7.3 (± 0.1)	8.2 (± 0.3)	9.2 (± 0.1)	4.3 (± 0.1)	5.5 (± 0.1)	6.1 (± 0.8)	7.1 (± 0.2)	7.5 (± 0.1	4.1 (± 0.1)	5.1 (± 0.1)	5.1 (± 0.3)	4.0 (± 0.3)	2.9 (± 0.2)	4.1 (± 0.6)	5.1 (± 0.8)	5.1 (± 0.1)	4.9 (± 0.1)	2.9 (± 0.5)
Stenotrophomonas sp. (SS4)	4.3 (± 0.)	5.1 (± 0.3)	5.4 (± 0.5)	7.4 (± 0.4)	8.9 (± 0.2)	3.2 (± 0.2)	5.4 (± 0.1)	6.3 (± 0.7)	7.3 (± 0.3)	7.6 (± 0.2)	3.3 (± 0.3)	5.2 (± 0.7)	6.1 (± 0.3)	5.3 (± 0.1)	4.3 (± 0.3)	4.3 (± 0.5)	4.3 (± 0.5)	4.1 (± 0.5)	2.1 (± 0.6)	2.7 (± 0.4)
K. pneumoniae (CR2)	4.5 (± 0.4)	5.4 (± 0.1)	6.1 (± 0.7)	7.1 (± 0.7)	10.2 (± 0.4)	3.3 (± 0.1)	4.2 (± 0.5)	5.1 (± 0.7)	5.2 (± 0.4)	6.5 (± 0.2)	4.0 (± 0.1)	5.1 (± 0.4	6.2 (± 0.1)	6.2 (± 0.1)	2.2 (± 0.6)	ND	ND	ND	ND	ND
K. pneumoniae (CR3)	3.1 (± 0.7)	5.4 (± 0.4)	6.2 (± 0.6)	6.3 (± 0.1)	8.4 (± 0.2)	3.2 (± 0.4)	4.3 (± 0.1)	5.1 (± 0.3)	5.3 (± 0.4)	7.1 (± 0.3)	3.1 (± 0.5)	4.3 (±0.78)	5.3 (± 0.4)	5.3 (± 0.1)	6.5 (± 0.3)	2.5 (± 0.6)	3.8 (± 0.5)	2.5 (± 0.1)	1.6 (± 0.3)	1.3 (± 0.1)
Serratia sp. (CB2)	4.3 (± 0.2)	5.4 (± 0.3)	6.0 (± 0.4)	7.1 (± 0.5)	10.1 (± 0.5)	3.3 (± 0.4)	5.2 (± 0.2)	6.3 (± 0.2)	6.1 (± 0.4)	6.3 (± 0.3)	5.1 (± 0.2)	4.1 (± 0.1)	5.0 (± 0.3)	3.3 (± 0.1)	2.3 (± 0.45)	ND	ND	ND	ND	ND
B.subtilis (CB3)	4.3 (± 0.6)	5.2 (± 0.1	6.3 (± 0.2)	8.4 (± 0.1)	10.0 (± 0.1)	3.0 (± 0.1)	5.1 (± 0.1)	6.4 (± 0.1)	6.4 (± 0.5)	7.2 (±0.3)	4.2 (± 0.5)	5.2 (± 0.7)	5.0 (±0.1)	4.1 (± 0.4)	4.2 (± 0.5)	2.5 (± 0.6)	3.7 (± 0.7)	2.5 (± 0.5)	2.5 (±0.3)	1.9 (± 0.1)
Klebsiella sp.(CB4)	4.5 (± 0.2)	5.4 (± 0.3)	5.1 (± 0.5)	7.1 (± 0.6)	9.2 (± 0.7)	3.3 (± 0.5)	4.2 (± 0.3)	5.1 (± 0.2)	6.2 (± 0.7)	7.5 (± 0.5)	3.0 (±0.5)	4.1 (± 0.7)	5.2 (± 0.5)	3.2 (± 0.6)	2.2 (± 0.5)	ND	ND	ND	ND	ND
Serratia sp.(OR3)	3.3 (± 0.6)	5.4 (± 0.7)	7.0 (± 0.1)	8.1 (± 0.6	9.1 (± 0.1)	4.3 (± 0.1)	5.2 (± 0.1)	5.3 (± 0.1)	6.3 (± 0.1)	7.3 (± 0.1)	3.1 (±0.15)	4. (± 0.1)	5.0 (± 0.1)	3.3 (± 0.1)	2.3 (± 0.1)	ND	ND	ND	ND	ND
S. saprophyticus (OR5)	3.3 (± 0.6)	5.2 (± 0.1)	6.3 (± 0.1)	7.4 (± 0.1)	9.0 (± 0.1)	3.0 (± 0.1)	5.1 (± 0.1)	6.4 (± 0.1)	6.4 (± 0.1)	6.3 (± 0.1)	3.2 (± 0.1)	4.2 (±0.1)	5.0 (± 0.1)	4.1 (± 0.4)	3.2 (± 0.5)	2.5 (± 0.1)	3.7 (± 0.8)	2.5 (± 0.6)	2.5 (± 0.1)	1.9 (± 0.1)
Klebsiella sp.(OR7)	4.1 (± 0.7)	5.5 (± 0.2	6.4 (± 0.1)	7.1 (± 0.5)	9.3 (± 0.7)	4.2 (± 0.1)	5.2 (± 0.1)	6.1 (± 0.1)	7.2 (± 0.1)	8.3 (± 0.1)	3.1 (± 0.1)	4.5 (± 0.6)	5.3 (± 0.4	3.3 (± 0.1)	3.3 (± 0.3)	ND	ND	ND	ND	ND
S. marcescens (CD1)	4.0 (± 0.5)	5.3 (± 0.1)	7.2 (± 0.1)	8.3 (± 0.4	9.2 (± 0.4)	5.3 (± 0.7)	5.2 (± 0.1)	6.3 (± 0.4)	7.3 (± 0.2)	8.3 (± 0.3)	3.2 (± 0.1)	5.3 (± 0.1)	5.1 (± 0.6)	3.5 (± 0.1)	2.3 (±1.6)	ND	ND	ND	ND	ND
Bacillus sp.(CD2)	3.2 (± 0.8)	5.2 (± 0.1)	6.2 (± 0.1)	6.2 (± 0.35	8.7 (± 0.6)	3.3 (± 0.6)	4.3 (± 0.8)	4.7 (± 0.5)	5.9 (± 0.2)	7.3 (± 0.1)	3.2 (± 0.1)	3.1 (± 0.4)	5.5 (± 0.1)	3.1 (± 0.7	3.4 (± 0.1)	ND	ND	ND	ND	ND
K. pneumoniae (SV1)	4.3 (± 0.4)	5.3 (± 0.1)	6.3 (± 0.1)	7.3 (±0.19)	9.3 (± 0.6	3.3 (± 0.5)	5.1 (± 0.5)	6.1 (± 0.1)	7.2 (± 0.1)	8.3 (± 0. 2)	3.1 (± 0.7)	5.1 (± 0.4)	4.5 (± 0.5)	4.3 (± 0.4)	3.5 (± 0.6)	ND	ND	ND	ND	ND
A.lipoferum (Az 204) **	4.1 (± 0.1)	5.4 (± 0.3)	6.4 (± 0.1)	7.3 (± 0.3)	8.4 (± 0.1)	3.3 (± 0.1)	4.3 (± 0.2)	5.5 (± 0.1)	6.5 (± 0.1)	7.5 (± 0.8)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are mean (± SE) (n=3); *hours after inoculation; **standard culture; ND-not detected

Table 4. Effect of temperature on the survival of diazotrophs isolated from the tissues of different grass species

Isolates	Population (log cfu ml^{-1}) at different level of temperature																			
	40 °C					45 °C					50 °C					55 °C				
	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h	0h	12h	24h	36h	48h
Pseudomonas sp.(CGE3)	4.1 (±0.9)	4.5 (± 0.1)	7.1 (± 0.3)	9.1 (± 0.1)	10.0 (± 0.1)	4.0 (± 0.1)	5.4 (± 0.5)	6.3 (± 0.1)	6.5 (±0.5)	7.3 (±0.6)	3.1 (±0.8)	4.3 (±0.1)	5.2 (±0.3)	4.3 (±0.4)	2.9 (± 0.1)	ND	ND	ND	ND	ND
Pantoea sp. (PRE2)	4.1 (± 0.3)	5.1 (± 0.6)	6.0 (± 0.1)	6.2 (±0.3)	8.3 (± 0.1)	4.2 (± 0.1)	5.1 (±0.1)	6.3 (±0.1)	6.5 (±0.5)	8.6 (±0.3)	4.1 (±0.9)	5.3 (±0.1)	5.3 (±0.3)	5.1 (±0.5)	2.8 (± 0.1)	ND	ND	ND	ND	ND
Serratia sp. (CRE9)	4.1 (±0.8)	5.1 (± 0.1)	5.3 (± 0.3)	7.1 (± 0.3)	8.0 (± 0.8)	3.1 (± 0.6)	5.0 (± 0.1)	6.1 (± 0.3)	7.2 (± 0.2)	8.0 (± 0.5)	3.4 (± 0.4)	5.1 (± 0.1)	6.0 (± 0.1)	5.1 (± 0.1)	4.4 (± 0.1)	4.3 (±0.1)	4.6 (±0.5)	4.1 (±0.1)	3.0 (±0.2)	3.1 (±0.1)
Pseudomonas sp.(CRE10)	3.0 (± 0.7)	6.2 (± 0.6)	7.2 (± 0.1	8.1 (± 0.1)	9.4 (± 0.4)	4. 1 (± 0.1)	5.5 (±0.4)	3.1 (± 0.1)	5.1 (± 0.3)	7.0 (± 0.5)	3.2 (± 0.4)	5.0 (± 0.1)	5.2 (± 0.1)	4.1 (±0.1)	3.3 (± 0.4)	ND	ND	ND	ND	ND
Bacillus sp. (CBE9)	4.2 (± 0.1)	5.3 (± 0.8)	6.0 (± 0.2)	7.1 (± 0.1)	9.2 (± 0.2)	3.2 (± 0.1)	4.1 (± 0.4)	5.1 (± 0.1)	5.3 (± 0.1)	6.1 (± 0.1)	4.5 (± 0.3)	5.4 (± 0.1)	6.3 (± 0.1)	6.2 (± 0.1)	3.1 (± 0.1)	2.5 (±0.1)	4.2 (±0.1)	3.1 (±0.1)	2.1 (±0.1)	1.3 (±0.5)
Enterobacter sp.(ORE7)	3.1 (±0.2)	5.1 (± 0.4)	6.3 (± 0.2)	7.5 (±0.7)	8.2 (±0.1)	3.5 (±0.1)	4.1 (±0.5)	5.1 (± 0.3)	5.3 (± 0.8)	7.3 (± 0.6)	3.0 (± 0.7)	4.0 (± 0.5)	5.1 (± 0.1)	5.1 (± 0.1)	5.5 (± 0.1)	ND	ND	ND	ND	ND
P.agglomerans (ORE9)	4.6 (± 0.5)	5.4 (± 0.)	6.3 (± 0.5)	7.4 (± 0.3)	9.2 (± 0.1)	3.2 (± 0.4)	5.2 (± 0.1)	6.2 (± 0.1)	6.4 (± 0.3)	7.5 (± 0.4)	5.1 (± 0.2)	4.1 (± 0.1)	5.0 (± 0.1)	3.1 (± 0.4)	1.6 (± 0.4)	ND	ND	ND	ND	ND
E. sacchari (SVE9)	4.0 (±0.5)	5.5 (± 0.4)	6.6 (± 0.3)	7.3 (± 0.1)	8.5 (± 0.2)	3.0 (± 0.1)	5.0 (± 0.1)	6.0 (± 0.1)	6.3 (± 0.1)	6.6 (± 0.5)	3.1 (± 0.1)	4.2 (± 0.1)	5.3 (± 0.1)	4.5 (± 0.2)	4.4 (± 0.1)	2.5 (±0.1)	4.1 (±0.5)	3.1 (±0.5)	3.1 (±0.1)	2.2 (±0.5)
G. diazotropicus (PAL5) **	5. 1 (± 0.1)	6.3 (± 0.1)	6.3 (±0.3)	7.1 (±0.1)	8.1 (±0.1)	3.1 (±0.4)	5.1 (±0.2)	5.9 (±0.2)	6.3 (±0.1)	7.4 (±0.3)	3.2 (±0.2)	4.3 (±0.1)	5.1 (±0.1)	4.4 (± 0.1)	3.0 (± 0.1)	ND	ND	ND	ND	ND

Values are mean (± SE) (n=3); *hours after inoculation; **standard culture; ND-not detected

Table 5. Effect of soil moisture potential on the growth of diazotrophs isolated from the rhizosphere of different grass species

Isolates	Moisture Potential (1BAR)					Moisture Potential (15BAR)				
	1DAI	15 DAI	30 DAI	45 DAI	60 DAI	1 DAI	15 DAI	30 DAI	45 DAI	60 DAI
Enterobacter sp.(BR1)	3.4 (± 0.4)	5.4 (± 0.4)	6.7 (± 0.5)	8.5 (± 0.1)	9.6 (± 0.4)	3.1 (± 0.2)	5.1(± 0.2)	6.3 (± 0.4)	8.4 (± 0.4)	9.6 (± 0.5)
Klebsiella sp.(CG1)	4.2 (± 0.3)	5.4 (± 0.3)	6.7 (± 0.4)	7.8 (± 0.3)	10.3 (± 0.2)	4.0 (± 0.1)	5.9 (± 0.2)	7.3 (± 0.1)	8.2 (± 0.1)	10.1 (± 0.6)
Enterobacter sp.(CG3)	3.8 (± 0.3)	5.7 (± 0.3)	7.8 (± 0.1)	8.9(± 0.3)	9.6 (± 0.4)	4.0 (± 0.4)	5.3 (± 0.1)	7.4 (± 0.1)	8.2(± 0.1)	10.8 (± 0.8)
Bacillus sp. (CG5)	3.2 (± 0.1)	5.9 (± 0.1)	6.8 (± 0.1)	7.8 (± 0.1)	10.2 (± 0.1)	3.1 (± 0.7)	5.0 (± 0.7)	5.3 (± 0.1)	7.4 (± 0.7)	9.5 (± 0.7)
Stenotrophomonas sp. (SS4)	3.4 (± 0.1)	5.4 (± 0.1)	6.9 (± 0.1)	7.9 (± 0.1)	9.7 (± 0.1)	4.1 (± 0.1)	5.3 (± 0.4)	6.3 (± 0.1)	7.4 (± 0.1)	9.4 (± 0.2)
K. pneumoniae (CR2)	3.5 (± 0.2)	5.6 (± 0.1)	6.4 (± 0.1)	7.8 (± 0.1)	9.8 (± 0.1)	3.3 (± 0.8)	5.5 (± 0.1)	6.2 (± 0.1)	6.3 (± 0.2)	8.4 (± 0.7)
K. pneumoniae (CR3)	3.5 (± 0.7)	6.2 (± 0.1)	7.4 (± 0.1)	8.3 (± 0.1)	9.4 (± 0.1)	4.0 (± 0.1)	5.3 (± 0.2)	6.0 (± 0.1)	7.0 (± 0.1)	10.0 (± 0.4)
Serratia sp. (CB2)	3.2 (± 0.6)	6.4 (± 0.1)	7.4 (± 0.1)	8.4 (± 0.4)	9.3 (± 0.6)	4.1 (± 0.4)	5.2 (± 0.1)	6.3 (± 0.1)	8.4 (± 0.1)	10.0 (± 0.9)
B.subtilis (CB3)	3.2 (± 0.4)	5.4 (± 0.1)	7.4 (± 0.1)	8.5 (± 0.6)	9.1 (± 0.1)	4.1 (± 0.6)	5.3 (± 0.1)	6.0 (± 0.1)	7.2 (± 0.7)	10.1 (± 0.7)
Klebsiella sp.(CB4)	3.1 (± 0.4)	5.2 (± 0.1)	6.3 (± 0.1)	7.4(± 0.1)	9.2 (± 0.8)	4.1 (± 0.1)	5.5 (± 0.3)	5.4 (± 0.1)	7.0 (± 0.8)	10.0 (± 0.6)
Serratia sp.(OR3)	3.5 (± 0.3)	5.3 (± 0.1)	6.4 (± 0.1)	7.1 (± 0.9)	9.1 (± 0.1)	3.0 (± 0.3)	5.3 (± 0.1)	6.2 (± 0.1)	7.3 (± 0.2)	9.2 (± 0.1)
S. saprophyticus (OR5)	3.4 (± 0.1)	5.6 (± 0.1)	6.2 (± 0.1)	7.3(± 0.8)	9.5 (± 0.1)	3.2(± 0.1)	5.1 (± 0.4)	7.2 (± 0.1)	8.2 (± 0.3)	9.2 (± 0.1)
Klebsiella sp.(OR7)	3.2 (± 0.1)	4.9 (± 0.1)	6.1 (± 0.1)	8.1 (± 0.1)	9.7 (± 0.1)	4.1 (± 0.1)	5.5 (± 0.1)	6.9 (± 0.9)	7.6 (± 0.4)	10.1 (± 0.1)
S. marcescens (CD1)	3.4 (± 0.3)	4.9 (± 0.1)	6.3 (± 0.1)	8.1 (± 0.6)	9.3 (± 0.2)	4.1 (± 0.1)	5.2 (± 0.5)	7.1 (± 0.1)	8.1 (± 0.6)	9.2(± 0.1)
Bacillus sp.(CD2)	3.1 (± 0.1)	4.5 (± 0.4)	6.7 (± 0.1)	8.5 (± 0.1)	9.4 (± 0.6)	3.2 (± 0.9)	5.2 (± 0.1)	6.1 (± 0.5)	6.3 (± 0.6)	9.4 (± 0.4)
K. pneumoniae (SV1)	3.0 (± 0.2)	4.3 (± 0.5)	6.4 (± 0.1)	8.5 (± 0.1)	9.5 (± 0.5)	4.4 (± 0.8)	5.3 (± 0.8)	6.3 (± 0.8)	7.4 (± 0.1)	9.4 (± 0.5)
A.lipoferum (Az 204) **	3.2 (± 0.3)	4.3 (± 0.3)	5.7 (± 0.1)	8.3 (± 0.1)	9.5 (± 0.1)	4.0 (± 0.1)	5.2 (± 0.1)	6.2 (± 0.1)	7.14(± 0.1)	8.3 (± 0.1)

Values are mean (± SE) (n=3); *hours after inoculation; **standard culture

Table 6. Effect of soil moisture potential on the growth of diazotrophs isolated from the tissues of different grass species

Isolate	Moisture Potential (1BAR)					Moisture Potential (15BAR)				
	0 DAI	15 DAI	30 DAI	45 DAI	60 DAI	0 DAI	15 DAI	30 DAI	45 DAI	60 DAI
Pseudomonas sp.(CGE3)	4.2 (± 0.7)	5.2 (± 0.2)	7.3 (± 0.2)	9.1 (± 0.7)	10.6 (± 0.4)	4.1 (± 0.1)	5.7(± 0.4)	7.9(± 0.1)	9.4(± 0.1)	10.8(± 0.1)
Pantoea sp. (PRE2)	4.7 (± 0.5)	5.4 (± 0.2)	6.1 (± 0.3)	6.2 (± 0.5)	8.6 (± 0.6)	4.2 (± 0.2)	5.9(± 0.4)	6.9(± 0.1)	7.3 (± 0.3)	8.0 (± 0.3)
Serratia sp. (CRE9)	4.5 (± 0.2)	4.9 (± 0.3)	5.3 (± 0.4)	7.3 (± 0.1)	8.4 (± 0.7)	4.2 (± 0.4)	5.6(± 0.3)	5.7(± 0.2)	7.7 (± 0.4)	8.9 (± 0.4)
Pseudomonas sp.(CRE10)	3.5 (± 0.3)	6.2 (± 0.7)	7.2 (± 0.5)	8.1 (± 0.9)	9.4(± 0.1)	3.0 (± 0.5)	6.7(± 0.5)	7.2(± 0.4)	8.5 (± 0.5)	9.8 (± 0.6)
Bacillus sp. (CBE9)	3.7 (± 0.3)	5.1 (± 0.6)	6.3 (± 0.7)	7.5 (± 0.7)	8.2 (± 0.1)	3.2(± 0.3)	5.9(± 0.3)	6.8(± 0.4)	8.9 (± 0.1)	8.4 (± 0.7)
Enterobacter sp.(ORE7)	3.8 (± 0.3)	5.0 (± 0.4)	6.0 (± 0.3	7.1 (± 0.5)	9.2 (± 0.8)	3.8 (± 0.4)	5.5 (± 0.9)	6.4 (± 0.2)	7.3 (± 0.1)	9.2 (± 0.9)
P.agglomerans (ORE9)	4.7 (± 0.2)	5.2 (± 0.3)	6.6 (± 0.4)	7.3 (± 0.4)	8.5 (± 0.4)	4.0(± 0. 1)	5.2 (± 0.4)	6.7 (± 0.5)	7.8 (± 0.1)	8.5 (± 0.3)
E. sacchari (SVE9)	3.5 (± 0.5)	5.0 (± 0.2)	6.2(± 0.1)	7.1 (± 0.3)	7.9 (± 0.3)	3.3(± 0. 2)	5.2 (± 0.2)	6.5(± 0.6)	7.4 (± 0.2)	7.9 (± 0.2)
G. diazotropicus (PAL5) **	3.4 (± 0.1)	5.3 (± 0.1)	5.3 (± 0.3)	7.1 (± 0.2)	8.1 (± 0.4)	3.3(± 0. 1)	5.3 (± 0.4)	5.9 (± 0.6)	7.5 (± 0.1)	8.4 (± 0.6)

Values are mean (± SE) (n=3), *DAI-Days after inoculation, **standard strain

ANNEXURE II

COMPOSITION OF VARIOUS MEDIA

Soil extract agar médium (Subba-Rao, 1977**)**

Glucose	-	10.0 g
K_2HPo_4	-	5.0 g
Soil extract	-	100 ml
Distilled water (volume to)	-	1000 ml
pH	-	7.5
Agar	-	20.0 g

Rose Bengal medium (Martin, 1950)

Glucose	-	10.0g
Peptone	-	5.0g
$KH_2 PO_4$	-	1.0g
Mg SO_4	-	0.05g
Streptomycin	-	30.0g
Agar	-	15.0g
Rose Bengal	-	0.035g
Distilled water	-	1000ml

Kenknights Agar Medium (Rangaswami, 1966)

Dextrose	-	1.0g
$KH_2 PO_4$	-	0.1g
$NaNO_3$	-	0.1g
KCl	-	0.1g
$MgSO_4$	-	0.1g
Agar	-	15.0g
Distilled water	-	1000ml

Total diazotroph medium (Döbereiner, 1989)

Glucose	-	0.5g
Malic acid	-	0.5g
Yeast extract	-	0.1g
$K_2 HPO_4$	-	0.5g

$FeSO_4$	-	0.05g
$MgSO_4$	-	0.02g
Na $MoO_4 2H_2O$	-	Trace
$CaCl_2$	-	0.2g
$H_3 BO_3$	-	0.15g
$ZnSO_4$	-	0.07g
$CuSO_4$	-	Trace
$MnCL_2$	-	Trace
Agar	-	20.0g
Distilled water	-	1000ml
pH	-	7.0
NFb medium (Döbereiner, 1989)		
Malic acid	-	5.0 g
K_2HPO_4	-	0.5 g
$MgSO_4 7H_2O$	-	0.2 g
NaCl	-	0.1 g
$CaCl_2$	-	0.02 g
Bromthymol blue0.5% in KOH 0.2 N	-	2 ml
Vitamin solution	-	1 ml
Micronutrient solution	-	2 ml
1.64% FeEDTA solution	-	4 ml
KOH	-	4.5 g
pH	-	6.8
Distilled water	-	1000ml
Agar	-	1.9 g
The vitamin solution(100ml)		
biotin	-	10 mg
pyridoxol-HCl	-	20 mg
Micronutrient solution (1000ml)		
$CuSO_4$	-	0.4 g
$ZnSO_4 7H_2O$	-	0.12 g

H_2BO_3	-	1.4 g
$Na_2MoO_42H_2O$	-	1.0 g
$MnSO_4H_2O$	-	1.5 g

JNFb medium (Kirchhof *et al*., 1997**)**

The medium called JNFb consisted of the same components as NFb-medium except following different ingredients

K_2HPO_4	-	0.6 g
KH_2PO_4	-	1.8 g
pH	-	5.8
Distilled water	-	1000ml
Agar	-	1.9 g

LGI medium (Reis *et al*., 1994)

Sucrose	-	5.0 g
K_2HPO_4	-	0.2 g
KH_2PO_4	-	0.6 g
$MgSO47H_2O$	-	0.2 g
$CaCl_2$	-	0.02 g
$Na_2MoO_42H_2O$	-	0.002 g
$FeCl_3$	-	0.01 g
Bromthymol blue 0.5% in KOH 0.2 N	-	5 mL
pH	-	6.0
Distilled water	-	1000ml
Agar	-	1.9 g

NFMM (Piao *et al*., 2005)

Sucrose	-	10.0 g
K_2HPO_4	-	0.4 g
KH_2PO_4	-	0.2 g
$MgSO47H_2O$	-	0.1 g
$CaCl_2$	-	0.01 g
$Na_2MoO_42H_2O$	-	0.002 g
$FeCl_3$	-	0.01 g

pH	-	7.2
Distilled water	-	1000ml
Agar	-	1.9 g
SM medium (Reinhold *et al*., 1985)		
Malic acid	-	5.0 g
KOH	-	4.5 g
KH_2PO_4	-	0.6 g
K_2HPO_4	-	0.4 g
$MgSO_4\,7H_20$	-	0.2 g
NaCl	-	0.1 g
$MnSO_4$- H_20	-	0.01 g
Na_2MoO_4- $2H_20$	-	0.002 g
Fe(III)-EDTA (0.66% (w/v) in H_2O)	-	10 ml
Biotin	-	0.1mg
Vitamin solution		10ml
pH	-	6.8
Distilled water	-	1000ml
Agar	-	1.9 g
Vitamin solution(1000ml)		
Biotin	-	200 mg
Calcium pantothenate	-	40 mg
Myoinositol	-	200 mg
Niacinamide	-	40 mg
Paminobenzoic acid	-	20 mg
Pyridoxine hydrochloride	-	40mg
Riboflavin	-	20 mg
Thiamine dichloride	-	4 mg

SSM medium (Reinhold *et al*., 1986)

Malic acid	-	5.0 g
KOH	-	4.8 g
NaCl	-	1.2 g
Na_2SO_4	-	2.4 g
$NaHCO_3$	-	0.5 g
$CaC1_2$	-	0.22 g
$MgSO_4$.$7H_20$	-	0.25 g
K_2SO_4	-	0.17 g
Na_2CO_3	-	0.09 g
Fe(III)-EDTA	-	0.077 g
K_2HPO_4	-	0.13 g
Biotin	-	0.1 mg
$MnCl_2$ - $4H_20$	-	0.2 mg
$CuCl_2$ $2H_20$	-	0.02 mg
$ZnC1_2$	-	0.15mg
H_3BO_3	-	0.20 mg
Na_2MoO_4. $2H_20$	-	2 mg
Agar	-	2.0 g
Distilled water	-	1000ml
pH	-	8.5

Rennie medium (Rennie, 1980)

Soution A

K_2HPO_4	-	0.8
KH_2PO_4	-	0.2
$Na_2FeEDTA$	-	28mg
$Na_2MoO_4.2H_2O$	-	25mg
Yeast extract	-	100mg
Mannital	-	3.0g
Sucrose	-	5.0 g
60% sodium acetate	-	0.5ml

Sodium malate	-	2.0 g
Semisolid medium agar	-	1.7 g
Distilled water	-	900ml
pH	-	7.0

Solution B

MgSo4	-	0.2g
Cacl2	-	0.06g
Distilled water	-	100

The solutions were autoclaved separately and mixed after cooling. Biotin and *Para* aminobenzoic acid (100µl each) filter sterilize separately were added at final concentrations of 5 and 10µg/l respectively. The combined solution was mixed with 1.25ml of plant extract that had been extracted with ethanol.

Cellulase activity test medium (Cotty *et al*., 1990)

O-phosphoric acid swollen cellulose	-	10.0 g
KH_2PO_4	-	2.0 g
NH_42SO_4	-	1.4 g
Urea	-	0.3 g
$MgSO4.7H_2O$	-	0.3 g
$FeSO_4$	-	8.0 mg
$MnSO_4$	-	1.6 mg
$CoCl_2$	-	2.0 mg
Agar	-	18.0 g
pH	-	7.0
Distilled water	-	1000ml

Pectinase activity test medium (Rautela and Cowling, 1966)

Pectin	-	5.0 g
KH_2PO_4	-	4.0 g
Na_2HPO_4	-	6.0 g
Yeast extract	-	1.0 g
Agar	-	18.0 g
pH	-	7.0
DW	-	1000 ml

Peptone water

Peptone	-	10.0g
NaCl	-	15g
Distilled water	-	1000ml
pH	-	7.0

Composition of Luria-Bertani (LB) medium (Melody, 1997)

Tryptone	-	10.0 g
Yeast extract	-	5.0 g
NaCl	-	5.0 g
Distilled water	-	1000 ml
pH	-	7.4
Agar	-	20.0 g

Succinate medium for siderophore production

Succinic acid	-	4.0 g
K_2HPO_4	-	3.0 g
Ammonium sulphate	-	0.2 g
Agar	-	18.0 g
Distilled water	-	1000 ml
pH	-	7.0

Chorom azurol s agar

Solution A (Dye solution)

Chorom azurols S	-	60.5mg
Distilled water	-	50.0ml

Solution B

1Mm $FeCl_3.6H_2O$ in 10 mm HCL	-	10ml

Solution C

Hexa deacl trimethyl ammonium bromide	-	72.9
Distilled water	-	40ml

Modified Fiss minimal medium

KH_2PO4	-	5.03 g
L-asparagine	-	5.03 g
Glucose	-	5.0 g
MgSO4	-	40 mg
$MnSO_4$	-	100 µg
$ZnCl_2$	-	500 µg
Distilled water	-	1000 ml

Pikovaskaya broth

Glucose	-	10.0g
$Ca_3(PO_4)$	-	5.0g
$(NH_4)_2 S0_4$	-	0.5g
KCl	-	0.2g
$MgSo_4.7H_20$	-	0.1g
$MnSO_4$	-	Trace
$FeSo_4$	-	Trace
Yeast extract	-	0.2g
Distilled water	-	1000 ml
pH	-	6.5

Sperberg's hydroxy apetite medium (Sperber, 1958)

Glucose	-	10.0 g
Yeast extract	-	0.2 g
$MgSO_4$	-	0.1 g
$(NH_4)_2SO_4$	-	0.1 g
KCl	-	0.2 g
Soil extract	-	200 ml
Agar	-	20.0 g
Tap water	-	1000 ml
K_2HPO_4 (10%)	-	40 ml

$CaCl_2$ (10%)	-	60 ml
pH	-	6.8

Soil extract was prepared by autoclaving soil:water in 1:1 ratio and filtering through country filter paper. K_2HPO_4 (10%) and $CaCl_2$ (10%) were prepared and sterilized separately. K_2HPO_4 followed by $CaCl_2$ was added to the medium just before plating.

Aleksandrov medium (Aleksandrov *et al.*, 1967)

Glucose	-	5.0g
$MgSo_4$	-	0.5g
$FeCl_2$	-	0.005g
$CaCl_2$	-	0.1g
$CaPo_4$	-	2.0g
Potassium aluminium silicate	-	2.0g
Agar	-	2.0 g
Distilled water	-	1000ml
pH	-	8.5

Zinc solubilizing bacterial medium (Bunt and Rovira, 1955)

Peptone	-	1.0g
Yeast Extract	-	1.0g
K_2HPO_4	-	0.4g
Ammonium sulphate	-	0.50g
Magnesium chloride	-	0.10g
Ferric chloride	-	0.10g
Soil Extract	-	250 ml
Zinc Oxide	-	1.00g
Distilled water	-	750ml
Agar	-	15.0g
pH	-	6.6- 7.0

Soil extract was prepared by pressure steaming 1000g of sieved garden soil mixed with 1000 ml of tap water in the autoclave for 30 min. A pinch of $CaCO_3$ was added after steaming and the supernatant was filtered through Whatman No: 3 filter paper.

N-free Plant Nutrient medium (Fahreus, 1957)

$CaCl_2$	-	0.1g
KH_2PO_4	-	0.12g
Na_2HPO_4	-	0.1G
$MgSO_4$	-	0.15g
Ferric citrate	-	0.005g
Trace element solution	-	1.0ml
Agar	-	20g
Distilled water	-	1000ml
pH	-	7.0

Trace element stock solution

Boric acid	-	2.86g
Manganous sulphate	-	2.03g
Zinc sulphate	-	0.22g
Copper sulphate	-	0.08g
Sodium molybdate	-	0.14g
Distilled water	-	1000ml

Antibiotics used for intrinsic antibiotic resistance test of diazotrophic isolates from selected grass species

Hi-Media Dodeca Universal III - DEO 008

Antibiotic	Symbol	Concentration (µg)	Antibiotic	Symbol	Concentration (µg)
Ampicillin	A	10	Cefaclor	Cj	30
Cefuroxime	Cu	30	Azithromycin	At	30
Cephadroxyl	Cq	30	Erythromycin	E	15
Augmentin	Au	30	Cefaperazone	Cs	75
Penicillin	P	10 units	Clariththromycin	Cw	15
Cephotaxime	Ce	30	Ciprofloxacin	Cf	5

* Concentration is in µg unless and otherwise specified

Publications

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Saline tolerant plant growth promoting diazotrophs from rhizosphere of bermuda grass and their effect on rice

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ABSTRACT

In this study, beneficial effects of multifaceted growth promoting isolates for rice were investigated under two different salt concentrations in pot culture conditions. Two most salt tolerant isolates (TRY2) *Serratia* sp. and *Bacillus* sp. (TRY4) were selected and their growth promoting characters were studied under slight and moderate NaCl concentration. Isolates *Serratia* sp. and *Bacillus* sp. were able to fix the nitrogen and solubilise phosphate, synthesise IAA, acc deaminase regardless of NaCl concentration in most cases, under conditions of salinity. In pot experiments, plant growth (plant height, dry weight, and chlorophyll content) was promoted by bacterial inoculation with 2.9 and 5.8 g NaCl/kg soil. In this study, uptake nutrients (N^+, P^+, and K^+) were increased regardless of NaCl concentration with inoculation of *Serratia* sp. and *Bacillus* sp. and uptake of Na^+ was reduced with treatments receiving 5.8 NaCl/kg soil with *Serratia* sp. and *Bacillus* sp. isolates used as inoculants as compared to control. The present observations showed that strains *Serratia* sp. and *Bacillus* sp. partially alleviated the saline stress in rice, likely through the integration of several mechanisms that improve the plant response.

Key words: *Bacillus* sp, Plant growth promoting diazotrophs, Rice, Salinity stress, *Serratia* sp.

Salinity is one of the most serious environmental problems influencing crop growth throughout the world. In India, out of an estimated area of 187.7 million ha of total degraded lands, 8.1 million ha are salt affected in which 3.1 million ha are in the coastal regions (Sziderics *et al.* 2007). In most saline soils, sodium chloride is the predominant salt species, and its effect can be observed by decreased productivity or plant death (Munns 2005). Most of the plants possess several mechanisms to decrease the negative effects of salinity including regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways (Parida and Das 2005).

Cynodon dactylon Pers. (Poaceae), a hardy perennial grass, is one of the most commonly occurring weeds in India. It is widely accepted that the rhizosphere of any plant species is a unique niche harboring diversified bacterial communities, which serve as potential resource for bioprospecting. The rhizosphere of plant species growing profusely under stress-conditions harbors novel diazotrophs to meet their nitrogen requirement as observed in salt marsh grasses such as Sp. artina alterniflora, *Juncus roemerianus* (Bagwell and Lovell 2000), ligotrophic habitant *Drosera villosa* (Albino *et al.* 2006) and desert growing *Lasiurus* grass (Chowdhury *et al.* 2009).

The beneficial roles of diazotrophs to plants include nitrogen-fixation, mineral solubilization, production of phyto hormones such as indole acetic acid (IAA) and cytokinins. By virtue of such attributes, pre-treatment of seeds with a suspension of *Azotobacter* was shown to improve seed germination and plant growth (Ravikumar *et al.* 2004). An increasing supply of N through dinitrogen fixation may increase crop production in saline habitats (Yao *et al.* 2010). Diazotrophic bacteria are also PGPR, because of their competitive advantage in C-rich and N-poor environments (Kennedy *et al.* 2004). Several reports revealed that inoculation with free living diazotrophs like *Azotobacter*, *Pseudomonas* and *Azosp. irillum* increased the yield of rice by 20–55% (Mirza *et al.* 2006) and a strain of diazotrophic *Burkholderia* increased the rice plant biomass by 69% (Kennedy *et al.* 2004). Some isolations including genus *Pseudomonas* and *Bacillus* have been shown to have capacity to promote the wheat growth in salinated soils of Uzbekistan (Nautiyal *et al.* 2008). In other work, Palomino *et al.* (2009) reported that *Bacillus subtilis* is a Gram-positive sporulating bacterium able to adapt to wide variations in osmotic and saline strength. Studies

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showed that inoculation with *Azosp.irillum* spp. increased plant growth and the K^+/Na^+ ratio of two maize cultivars (Hamdia *et al*. 2004). Moreover, Yao *et al* (2010) reported that inoculation with *Pseudomonas putida* promoted cotton growth and germination under conditions of salt stress. Considering the efficacy, input cost and environmental safety, use of chemical fertilizers for crop production in saline soil is not a sound proposition.

The objective of this study was to identify the multifaceted growth promoting salt tolerant diazotrophs from the rhizosphere of *Cynodon dactylon* and their effect on rice seedling in the presence and absence of salt stress under pot culture conditions.

MATERIALS AND METHODS

Isolation and screening of growth promoting diazotrophs

The soil samples were collected from the rhizosphere of *C. dactylon* at salinity affected agricultural fields of Trichy district, Tamilnadu on March, 2012. Diazotrophic microorganisms isolated using serial dilution technique (10^{-6} dilution) on selective N-free malate medium (NFM) (Piao *et al*. 2005) with 1% (w/v) NaCl concentration. After required incubation period, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies from rhizosphere soil samples picked from NFM plates and sub-cultured several times in same medium to obtain pure cultures and stored as glycerol stocks at -20º C. The salt tolerances of diazotrophic bacterial isolates were determined on free nitrogen media supplemented with different NaCl concentrations of 0-10%.

Identification of diazotrophs by 16S rRNA gene sequencing

Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2 (Weisburg *et al*. 1991) and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using PGMT vector supplied with TA cloning kit (Promega, USA) prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST).

Plant growth promoting features of isolates

Plant growth-promoting capabilities of the selected strains were studied in the presence and absence of NaCl (nitrogen fixation, phosphate solubilization, indole acetic acid, acc d synthesis and siderophore production).The nitrogen fixing capacity of the diazotrophic isolates was evaluated by estimating the acetylene reduction activity (ARA) (Bergersen 1980) using gas chromatograph (Chemito-7610) equipped with FID detector and Porapak N column (2m x 1/8"). Temperatures such as injector, detector, and column were maintained at 110°C, 120°C and 73°C, respectively. Nitrogen gas was used as carrier gas at the flow rate of 30 ml/min. After completion of ARA, the bacterial cells in the medium were evenly mixed and the protein concentration was measured (Lowry *et al*.1951). ARA results were expressed as n mole ethylene /mg/protein/h. An aliquot was taken from each pure culture for evaluation of plant growth promoting characteristics indole acetic acid (IAA) production was done using the method of Chandramohan and Mahadevan (1968). Solubilization of insoluble phosphates (Bunt and Rovira 1955) were also assayed. Siderophore production was checked using the Chrome Azurol S (CAS) agar plates (Dubey and Maheshwari 2004). ACC deaminase activity was measured by measuring the production of á-Ketobutyrate as described by Honma and Shimomura (1978).

Effects of NaCl and bacterial inoculation on rice growth

Pot experiments were conducted in order to evaluate the effect of NaCl and bacterial inoculation on growth of rice. Pots containing 400 g of dry-sterilized soil were supplemented to reach 0, 2.9 and 5.8 g NaCl/kg soil, which was prepared by adding 0, 1.08 and 2.25 g NaCl dissolved in 100 mL water. The treatment without exogenous addition of NaCl was considered as 0 g NaCl/kg soil concentration. Characteristics of the soil without added salt were pH (1:5 water)- 6.5, EC- 1.50 dS /m, organic matter -15 g /kg, available N-289 kg/ha, P-18.92 kg/ha, potassium - 134.34 kg/ha. Dehulled rice seeds var. *ADT43* were disinfected by soaking in 30% hydrogen peroxide and 70% ethanol for 10 min, and followed by rinsing several times in sterilized distilled water. The seeds were then pre-germinated in sterilized plain agar at room conditions for five days. For inoculum preparation, bacteria were grown in nutrient broth for 24 h at 37ºC, rinsed twice, and finally resuspended to the same initial volume using phosphate buffer pH7.0. Roots of seedlings, with the same size, were

submerged three times in bacterial suspension adjusted to OD_{600} = 1 and planted in each pot supplemented or not with NaCl. Seedlings submerged in sterilized water were used as a control. Biometric observations such as plant height and dry matter production were taken at 4 weeks after the inoculation. In addition, chlorophyll content of rice leaves was estimated by the method of Hiscox and Israelstam (1979).

Inorganic elements

Leaf tissues were separated after harvesting and air-dried at 70°C for 5 days. Dried materials were ground and then digested in H_2SO_4 for the determination of total nitrogen (Kjeldahl method) or in a ternary solution (HNO_3: H_2SO_4 : $HClO_4$ = 10:1:4 with volume) for the determination of P, K and Na (Mani *et al*. 2007).

RESULTS AND DISCUSSION

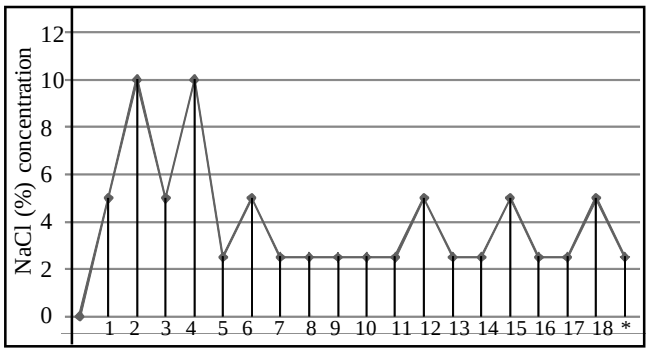
In the present study 18 pure cultures of diazotrophic organisms were isolated from saline soils and tested for tolerance of NaCl. Among the isolates tested, isolates C2 and C4 tolerated a higher content of NaCl (10%) than other isolates (Fig. 1). Due to their high tolerances, TRY2 and TRY4 were selected for further studies. Identification of isolates based on phenotypical and physiological criteria however was difficult, if the features displayed by a particular isolate are not fully identical with a described species. Utilization of PGPB has become a promising alternative to alleviate plant stress caused by salinity (Fu *et al*. 2010). Thus the molecular based method, 16S rDNA sequence analysis, was therefore chosen to identify the selected isolates. The isolates were identified as *Serratia* sp. (TRY2) and *Bacillus* sp. (TRY4) within the order Enterobateriales and Firmicutes, respectively (Table 1).

Table 1. Molecular characteristics of selected isolates

Code of the isolate	Length of 16sr DNA sequence (bp)	Most closely related organism	Similarity (%)
TRY2	1540	*Serratia* sp.	99
TRY4	1469	*Bacillus* sp.	99

Plant growth-promoting features

The isolates *Serratia* sp. and *Bacillus* sp. were able to reduce acetylene in both the presence and absence of NaCl. In the present study, the nitrogenase enzyme activity of the isolates ranged from 114 ± 4.63 to 136 ± 7.92 n moles of ethylene/mg of protein/h. The highest nitrogenase activity was exhibited by isolate *Bacillus* sp. with 2.9g NaCl concentration (136±7.92 n moles of ethylene/ mg of protein/ h) (Table 2).



TRY- 1 to 18; *Az 2 04

Fig. 1. Maximum salt tolerance level of diazotrophic isolates

The microbial synthesis of plant growth regulators is an important factor in soil fertility. Salt-tolerant IAA-producing bacterial strains *P. aureantiaca* and *P. extremorientalis* alleviated quite successfully the reductive effect of salt stress on percentage of germination (up to 79%), probably through their ability to produce IAA (Egamberdieva *et al.* 2008). They were able to produce indole-3-acetic acid (IAA) in saline conditions. In the present study, the maximum amount of IAA was produced by *Bacillus* sp. (18.8 ±1.0 µg/ml of sample) followed by *Serratia* sp. (14.8±0.8 µg/ml of sample) with 2.9 g/l NaCl concentration (Table 2).

Diazotrophic microorganisms showing phosphate solubilizing activity have beenreported. (Mayak *et al*. 2004). Phosphate solubilization activity was exhibited by both strains *Serratia* sp and *Bacillus* sp (Table 2). In the present study, the maximum amount of siderophore was produced by isolate *Bacillus* sp and *Serratia* sp with 2.9 NaCl concentration which produced 16.5±0.9 and 15.5±0.8 µg/ml of sample respectively. In other work, direct use of *P. putida* siderophores by plants has been demonstrated in many species, including dicot legumes such as peanut or monocots such as sorghum (Albino *et al*. 2006).

The results obtained demonstrated that the selected salt-tolerant bacterium containing ACC deaminase. *Pseudomonas fluorescens* strain TDK1 containing ACC deaminase activity enhanced the saline resistance in groundnut plants and increased yield as compared to plants inoculated with *Pseudomonas* strains lacking ACC deaminase activity (Saravanakumar and Samiyappan 2007). In the present study, maximum amount of ACC observed in *Bacillus* sp. (89.8 ± 1.1 nmoles of á-ketobutyrate /mg/h) followed by *Serratia* sp. (76.6 ± 2.3 nmoles of á - ketobutyrate/mg/h) with 5.8g/l NaCl concentration (Table

Table 2. Plant growth promoting rhizobacteria (PGPR) features of strains

Strain	NaCl (g/l)	[1]ARA	[2]IAA	[2]Phosphate solubilzation	[2]Siderophore production	[3]acc deaminase activity
Serratia sp.	0	117(±9.2)^{c}	11.7(±1.2)^{ab}	12.0(±0.9)^{ab}	11.0(±1.0)^{b}	13.9 (±1.0)^{d}
	2.9	122(±8.1)^{ab}	14.8(±0.8) ^{ab}	11.8(±0.7)^{ab}	15.5(±0.8)^{a}	34.8 (±1.4)^{c}
	5.8	118(±7.1)^{c}	10.3(±0.9)^{b}	14.8 (±1.2)^{a}	11.0(±0.6)^{b}	76.6 (±1.3)^{b}
Bacillus sp.	0	114(±4.6)^{ab}	11.8(±0.8) ^{b}	11.7 (±0.9)^{ab}	12.5(±0.8)^{b}	14.9 (±1.2)^{d}
	2.9	136 (±7.9)^{a}	18.8(±1.0)^{a}	12.8 (±0.7)^{ab}	16.5 (±0.9)^{a}	45.8 (±1.1)^{c}
	5.8	123 (±5.2)^{ab}	10.8(±1.0)^{b}	10.8 (±1.0)^{b}	10.5 (±1.0)^{b}	109.8(±1.2)^{a}

ARA: Acetylene reduction activity; IAA: Indole acetic acid; Values are mean (± standard error) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$). [1]n moles of ethylene/ mg of protein/h, [2]µg/ml of sample, [3]n moles of a-ketobutyrate/mg/h

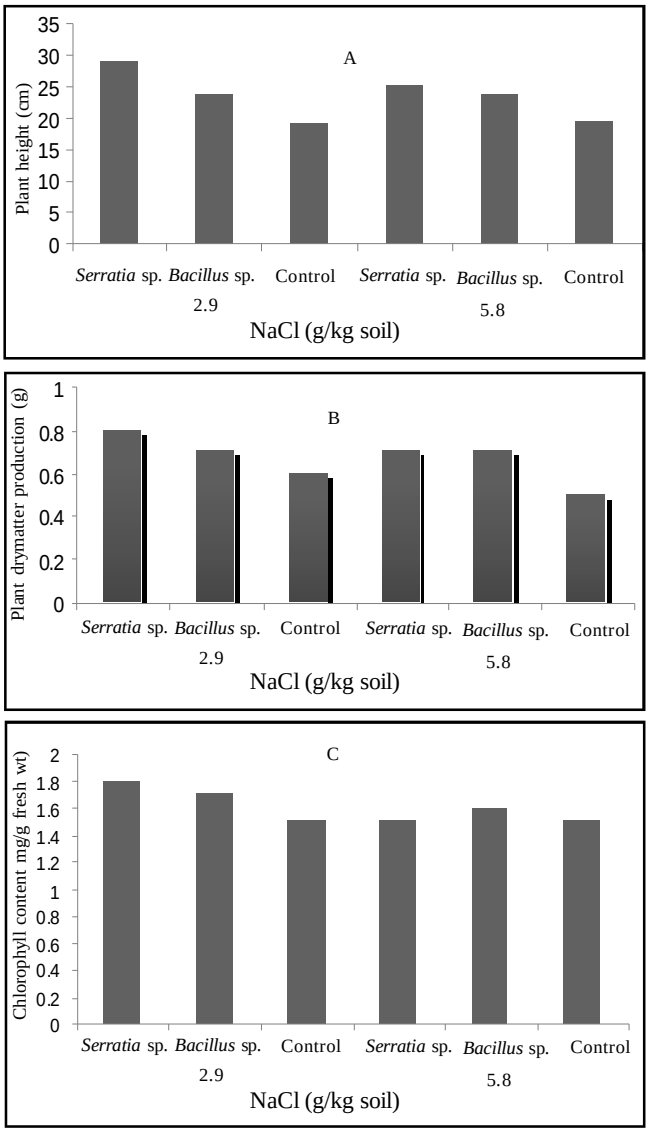


Fig 2. Effect of NaCl and diazotrophic PGPR on a) plant height, b) plant dry matter production and C) chlorophyll content of rice seedlings

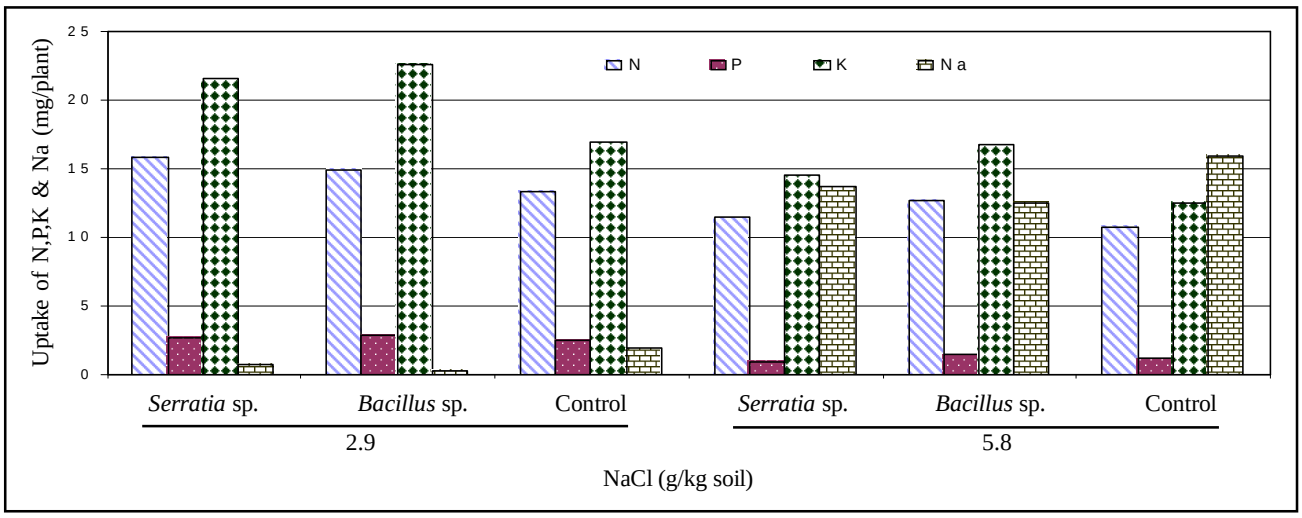
2). In other work, *Pseudomonas putida* UW4, which produces IAA and ACC deaminase, protected canola seedling from growth inhibition by high levels of salt (Mayak *et al*. 2004).

Rice growth and nutrient uptake

Electrical conductivities were 3.05 and 6.00 dS/m for 2.9 and 5.8 g NaCl/kg soil, respectively. Inoculation with strains *Serratia* sp. and *Bacillus* sp. increased plant growth, both normal and under saline stress. Similar work conducted by Mayak *et al*. (2004) that *Achromobacter piechaudii* having ACC deaminase activity significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl salt (up to 172 mM). In the present study, the treatment inoculation with strain *Bacillus* sp. in 1.5 g NaCl/kg soil, the increase in plant height (28.9± 1.3cm) and dry matter production (0.8 g) (Fig. 2). Salinity decreases carbon uptake by limiting photosynthesis, causing an over-reduction of photosynthetic electron chain, and redirecting the photon energy into processes that favour the production of reactive oxygen species (ROS) (Hichem *et al*. 2009). In our study, the maximum total chlorophyll (1.8± 0.3 mg) was observed in *Serratia* sp. with 2.9 NaCl g/kg. Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N poor environments (Kennedy *et al*. 2004). Hence inoculation with diazotrophic bacteria might improve crop growth and productivity in such soils.

The PGPR strains varied greatly in their effect on the concentration of major mineral nutrients in rice leaves under soil salinity conditions. The N, P, K, and Na uptake per plant in the soil salinity treatment were significantly decreased compared to the non-salinity treatment (Fig. 3). The concentration of major cations in the non-salinity treatment was increased more with the PGPR treatment (*Serratia* sp. and *Bacillus* sp.) than the control, but Na^{+}

Fig. 3 Effect of NaCl and diazotrophic PGPR on uptake N, P, K and Na



uptake under soil salinity were decreased in the treatment inoculated with (*Serratia* sp.) and (*Bacillus* sp.) strains. Among the isolates, *Bacillus* sp. caused a lower uptake of Na (22.5 ± 0.6 mg) compared to *Serratia* sp. (Fig. 3). Our findings clearly showed that two isolates of *Serratia* sp. and *Bacillus* sp. were capable of exerting multifaceted beneficial plant-growth promoting activities under moderate saline conditions. These isolates could serve as potential bioinoculants for meeting the nutritional requirement of the crop plants in an eco-friendly and cost effective manner in saline soil conditions.

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Isolation of elite diazotrophic bacterial isolates from *Cyanodon dactylon* rhizosphere of saline soils

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Abstract

In the present study, out of sixty rhizosphere diazotrophic isolates, twenty seven isolates from rhizosphere recorded positive in N- free medium. Isolates showing growth in N- free medium were subjected to ammonia production and total nitrogen concentration measurement. Among the diazotrophic isolates CD6 and CD12 were found to produce the maximum amount (14.90 ± 0.12 mg N g^{-1} of malate) of nitrogen and ammonia (4.3±0.26 mg ml^{-1}) respectively. The nitrogenase enzyme activity of the isolates ranged from 45.29 ±0.03 to 174.25 ±0.89 n moles of ethylene mg^{-1} of protein h^{-1}. The highest nitrogenase activity was exhibited by isolate CD6 (174.25 ±0.89 n moles of ethylene mg^{-1} of protein h^{-1}). The maximum amount of IAA and GA was produced by CD6 (26.7 ±0.25µg g^{-1} protein and CD20 (23.7µg g^{-1}protein) respectively.

In the present study the capability of the 8 isolates to solubilize insoluble forms of phosphorus and zinc were tested. Among the dizotrophic isolates, the isolate CD20 recorded the maximum production of siderophore and HCN (60.61 µg mg^{-1} dry weight of cell of catechol type and 61.8±1.18 µg ml^{-1}) respectively. The antagonistic activity of all the diazotrophic isolates against 2 rice pathogenic organism viz., sheath rot (R.solani) and blast (P.oryzae) was evaluated and 7 isolates were effective against 2 selected pathogens. The pot culture experiment results revealed that there was a significant increase in biomass, shoot length, root length, nitrogen content of diazotrophic isolates when compared with uninoculted control. Out of eight isolates tested in the present investigation, two efficient isolates viz., CR6 and CR20 were selected for 16 srRNA sequencing, isolates belong to Serratia sp and Klebsiella pneumoniae within the order Enterobateriales.

Keywords: Saline soils, *Cyanodon dactylon,* Diazotrophs, PGPR and16s rDNA sequencing.

Introduction

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals and pesticides are major limiting factors in crop production because they affect almost all plant functions. Salinity is one of the most serious environmental problems influencing crop growth throughout the world [32]. In India, out of an estimated area of 187.7 million ha of total degraded lands, 8.1 million ha are salt affected in which 3.1 million ha are in the coastal regions[41]. However, in these conditions, there are plant populations successfully adapted and evolutionarily different in their strategy of salt tolerance. N input for crop production is very important which is possibly by an increasing supply of N though dinitrogen fixation may increase crop production in saline habitats. Salt tolerant symbiotic nitrogen fixers like *Rhizobium* increase crop productivity in saline soils[43]. Considering the efficacy, input cost and environmental safety, the use of chemical fertilizers for crop production in coastal saline soil is not a sound proposition.

Cynodon dactylon Pers. (Poaceae), a hardy perennial grass, is one of the most commonly occurring weeds in India. It is a fast growing graminaceous plant and naturally adopted saline conditions. Generally graminaceous plants survive extreme environment conditions and grow even limited bioavailable inorganic nutrients and those microorganisms associated with such plants would possess higher adaptive mechanisms to cope with various stress conditions (viz. starvation, high osmolarity, high temperature, desiccation).

Hence, it is widely accepted that the rhizosphere of any plant species is a unique niche harboring diversified bacterial community which serves as potential resource for bioprospecting. The rhizosphere of plant species growing profusely under stress-conditions harbors novel diazotrophs meets their nitrogen requirement as observed in salt marsh grasses such as *Spartina alterniflora*, *Juncus roemerianus*[4], ligotrophic habitant *Drosera villosa*[2] and desert growing *Lasiurus* grass[14].

Material and Methods

Soil sampling and analysis: Rhizosphere and non rhizosphere soil from *C.dactylon* were collected from salinity affected areas of Trichy district, Tamilnadu on March, 2012. Plants were uprooted carefully and the soil adhering to the root was separated in a sterile Petri dish and mixed thoroughly so as to make a composite sample for microbiological analysis. The non-rhizosphere (bulk) soils were collected in the same location at a depth of 0-30 cm where the influence of the root of plants was not there. The samples were used immediately for preliminary analyses and stored at 4°C in refrigerator for further studies[28]. The soil pH, EC, total macro -nutrients including nitrogen, phosphorus

and potassium were analysed by following standard procedures.

Arginine ammonification rate was measured by the method of Alef and Kleiner[3] using an incubation period of 3 h and a temperature of 25±8^{0}C. Fluorescein diacetate hydrolysis rate was estimated as described by Schnurer and Rosswall[38] using an incubation period of 3 h at 24±8^{0}C. Potential N mineralization was determined in an aerobic incubation for 10 days at 25±8^{0}C. For microbiological analysis of soil, total bacteria, fungi and actinobacteria were enumerated using serial dilution plate technique [26].

Isolation of diazotrophs: Diazotrophic microorganisms were enumerated and isolated using serial dilution technique on selective N-free malate medium (NFM) [27]. Aliquots (0.1 ml) from the samples serially diluted up to 10^{-6}were added to the media in petri plates and kept in an incubator at 30 ^{0}C. Five days after incubation, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies from rhizosphere soil samples were picked from NFM plates and sub-cultured several times in same medium to obtain pure cultures and stored as glycerol stocks at -20 ^{0}C.

Preliminary screening for diazotrophy of the isolates: The diazotrophs isolated from rhizospheres of *C.dactylon* were tested for their ability to grow in N-free medium[9] and those isolates which had positive value in N- free medium were further analyzed for total nitrogen content[19] and ammonia production[11].

Secondary screening for diazotrophy of the isolates

Acetylene Reduction Activity (ARA): The nitrogen fixing capacity of the diazotrophic isolates was evaluated by estimating the Acetylene Reduction Activity (ARA) following the standard procedure[6]. Twenty five ml of modified N-free malate broth was prepared in 100 ml vials. The vials were inoculated with the cultures and were incubated under static conditions in an incubator at 28 ± 2°C. After 5 days of growth, the cotton plugs were replaced by suba-seal septa and capped with aluminium cap. The head space volume was replaced by 5% (v/v) acetylene which was generated from calcium carbide and water. The vials were incubated for 24 h at room temperature. After incubation, 1 ml of gas sample was withdrawn and injected into the Gas chromatograph (Chemito-7610) equipped with FID detector and Porapak N column (2m x ⅛"). Temperatures such as injector, detector and column are maintained at 110°C, 120°C and 73°C respectively.

Nitrogen gas was used as carrier gas at the flow rate of 30 ml min^{-1}. After completion of ARA, the bacterial cells in the medium were evenly mixed and the protein concentration was measured[22]. ARA results were expressed as n mole ethylene mg^{-1} protein h^{-1}. Those cultures which showed nitrogenase activity were selected for further studies.

Screening of diazotrophs for Multifaceted-Plant Growth promoting activities: An aliquot was taken from each pure culture for evaluation of Plant Growth Promoting characteristics. Indole Acetic Acid (IAA) and Gibberellic acid (GA_3) production was done using the method of Chandramohan and Mahadevan[12] and Borrow et al[7] respectively. Solubilization of insoluble phosphates [21]and zinc [8]were also assayed.

Siderophore production was checked using the Chrome Azurol S (CAS) agar plates[15]. Hydrogen cyanide (HCN) production by the diazotrophic isolates was assed using method of Sadasivam and Manickam[35]. The antagonistic potential of the isolates was determined against *Rhizoctonia solani* and *Pyricularia oryzae* by dual-culture method [30].

Pot culture experiment: Based on the N fixation and PGPR studies, the efficient isolates were taken for pot culture experiments to study the effect on biometric characteristics on rice seedlings. The surface sterilized rice seeds (ADT 43) were taken for the gnotobiotic study. The seeds were treated with 10^9ml^{-1} of respective inoculums and sown. For each isolates, 3 replications were maintained. An uninoculated plant served as control. Biometric observations were taken at 90 days after the inoculation.

Identification of diazotrophs by 16S rRNA gene sequencing: Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2[42] and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using PGMT vector supplied with TA cloning kit (Promega, USA) prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products was carried out on an Applied Biosystems (Model 3100) automated sequencer.

The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/ BLAST). The phylogenetic tree was constructed with existing 16S rRNA gene sequences from related eubacteria obtained from NCBI GenBank database by neighbor-joining method of Saitou and Nei[36] using MEGA 5.0 software[39].

Results and Discussion

Soil microorganisms play an important role in soil processes that determine plant productivity. Rhizosphere microbial communities are influenced by the plant exudates, roots as mechanical support and competition for nutrients. Equally, plants are affected by rhizosphere microbial communities through their participation in fast

soil nutrients cycle, water dependence and growth promoting metabolites[10].

The results of the present study confirmed that the rhizosphere had predominant influence on physico-chemical as well as biological properties of the soil. High pH, high Electrical conductivity and low organic carbon, low available N medium P and high K were reported in both rhizosphere soil. Generally, the pH was slightly lower in the rhizosphere (8.46 ± 0.02) than in the non-rhizosphere (8.46 ± 0.02). The electrical conductivity was lower to some extent in the rhizosphere. Both soils properties were on par with each other with slight variations. Rietz and Haynes[33] reported that there was a significant negative exponential relationship between EC and microbial biomass C.

The availability of all the three major nutrients viz. nitrogen, phosphorous and potassium also seemed to be considerably higher in the rhizosphere region (311 ± 15, 17.7 ± 2.79 and 265 ± 22Kg/ha) respectively. Higher microbial population was recorded in rhizospere soil of *C.dactylon* (Table 1). Similarly soil biological properties such as FDA and ADA were also higher in rhizosphere soil (14.09 ±0.30 µg flurescein g^{-1} and 4.4 ±0.17 (g g^{-1} soil h^{-1}) of *C.dactylon.* (Table 1). FDA hydrolysis rate is widely accepted as an accurate and simple method for measuring total microbial activity in soils[1] because FDA hydrolysis is mediated simultaneously by protease, esterase and lipase and it can reflect the activities of these enzymes in soil[38].

Arginine ammonification rate is an indicator of microbial activity in soil since most heterotrophs possess endocellular ammonifying capacity and its rate has been found to be closely correlated with soil microbial biomass and its activity[3]. The inverse relationship between the FDA hydrolysis rate, arginine ammonification rate and EC is in accordance with Rietz and Haynes[33]. The remarkably low FDA hydrolysis rate and arginine ammonification rate in soils with high salinity indicate the inhibitory effects that salinity inflicts on soil microbial activity.

In the present study, out of sixty rhizosphere diazotrophic isolates, 27 isolates from rhizosphere recorded positive in N- free medium. Isolates showing growth in N- free medium were subjected to ammonia production and total nitrogen concentration measurement. Total N determination using micro Kjeldhal distillation apparatus exhibited variation from 1.1 ± 0.01 to 14.90 ± 0.12 mg N g^{-1} of malate. Among the diazotrophic isolates CD6 was found to produce the maximum amount (14.90 ± 0.12 mg N g^{-1} of malate) of nitrogen and the least amount was produced by CD14 (1.1 ± 0.01 mg N g^{-1} of malate) of nitrogen (Table 2).

The isolate CD12 produced maximum quantity of ammonia (4.3±0.26 mg ml^{-1}) and the least amount of ammonia was produced by (3.1±0.12 mg ml^{-1}) isolates (Table 2). Rao et al[31] isolated nitrogen fixing organisms from partially decomposed municipal solid waste and the nitrogen fixing ability of the isolates were analyzed by microkjeldahl method. The confirmation test for nitrogen fixing ability was done by gas chromatographic analysis by acetylene reduction assay and the results are reported in table 3. The nitrogenase enzyme activity of the isolates ranged from 45.29 ±0.03 to 174.25 ±0.89 n moles of ethylene mg^{-1} of protein h^{-1}. The highest nitrogenase activity was exhibited by isolate CD6 (174.25 ±0.89 n moles of ethylene mg^{-1} of protein h^{-1}).

The standard culture *Azospirillum lipoferum* (AZ 204) showed the ARA activity (45.29 ±0.03) n moles of ethylene mg^{-1} of protein h^{-1}). Recently, Forchetti et al[18] isolated endophytic bacteria *Achromobacter xiloxidans* and *B. pumilus* from sunflower (*Helianthus annuus* L.) roots and both two strains were capable to grow in chemically defined medium without nitrogen source through the biological nitrogen fixation.

In the recent decades there has been increasing evidence that besides N_2–fixation, synthesis and export of phytohormones by the N_2–fixing bacteria may play an important role in the observed plant growth promotion. Out of 11 diazotrophic isolates, 7 isolates were able to produce the IAA. The maximum amount of IAA was produced by CD6 (26.7 ±0.25µg ml^{-1}) followed by CD 20 (24.6±0.23µg ml^{-1}). The highest amount of GA was produced by CD20 (23.7µg ml^{-1}) followed by CD6 which produced 22.4 ±0.23µg ml^{-1}. From a point of view of salinity condition regulated in plant-associated rhizobacteria, some isolations including genus *Pseudomonas* and *Bacillus* have been shown to have capacity to promote the wheat growth in salinated soils of Uzbekistan[16].

Similar results were reported in India in groundnut (*Arachis hypogaeae*) inoculated with *Pseudomonas fluorescens*, according to Saravanakumar and Samiyappan[37]. In other interesting work, Mayak et al[24] showed that *Achromobacter piechaudii* inoculation in tomato seedlings promoted the plant growth and conferred resistance to salt stress. The ability of the diazotrophic isolates to solubilize insoluble mineral salts of phosphorus and zinc was determined by observing the clearing zone produced in the respective medium and the results are presented in table 4. One of the various mechanisms by which rhizobacteria promote plant growth is by solubilization of insoluble minerals.

Hence, in the present study the capability of the 8 isolates to solubilize insoluble forms of phosphorus (tri calcium phosphate) and zinc (zinc oxide) were tested. Among the dizotrophic isolates, the isolate CD20 recorded with the maximum production of siderophore and HCN (60.61 µg mg^{-1} dry weight of cell of catechol type and 61.8±1.18 µg ml^{-1}) respectively. The antagonistic activity of all the diazotrophic isolates against 2 rice pathogenic organism viz. sheath rot (*R.solani)* and blast (*P.oryzae*) were evaluated (Table 4). Among the 11 cultures, only 7 isolates

were effective against 2 selected pathogens. Similar results were reported by Lucy et al[23] who proved that diazotrophs have an ability to produce IAA and solubilize insoluble phosphorus, antibiotic activity and also that it was able to colonize cereal roots effectively.

The effect of inoculated diazotrophic isolates on the growth parameters of rice was studied and the results are presented in table 5. Maximum biomass 0.09 g of rice seedlings was recorded on 30 DAI due to the inoculation of CD6 and CD20. It was observed that all the isolates and standard strain significantly increased the root and shoot length of rice seedlings when compared to control. The maximum shoot length (19.56 cm) and root length (9.30 cm) of rice seedlings were observed on CD6 at 30 DAI. It was observed that all the isolates and standard strain have significantly increased the nitrogen content of rice seedlings when compared to control.

The maximum nitrogen content was recorded in rice seedling inoculation with CD20 (1.88%) followed by CD (1.88 %). Free living diazotrophs have been reported to improve nutrient uptake efficiency and to fix N_2 through associative and endophytic associations with graminaceous plants[5].The results revealed that there was a significant increase in biomass, shoot length, root length, nitrogen content and enhanced bacterial communities like *Azospirillum,* P solublizers, total diazotrophs in rhizosphere of rice seedlings. This may be due to the auxin production, more nutrient (P and Zn) availability and nitrogen fixation and pathogen control by elite isolates. Out of eight isolates tested in the present investigation, two efficient isolates viz. CD6 and CD20 were selected for16 s rRNA sequencing based on multiple plant growth promoting activity compared to other cultures and standards.

Based on their 16S rDNA gene sequences, isolates were classified as gammaproteobacteria (CD6 and CD20) with members of the genus *Serratia* and *Klebsiella* with 98% sequence similarity (Fig. 1 and table 6). The CD6 and CD20 isolates belong to *Serratia sp* and *Klebsiella pneumoniae* within the order Enterobateriales. Trees constructed with neighbor joining and maximum parsimony provided support for the coherence of these clusters. Diverse species of *Serratia* have been isolated from rice rhizosphere[34] and rice seed[25]. It has been also found as an endophytic colonizer of rice[40].

Furthermore, the phosphate solubilization property and the presence of the nitrogen fixation gene of *S. marcescens* were demonstrated[23]. Due to chitinase activity inducing systemic resistance in plants[29], S. *marcescens* has been also used as biocontrol agent. Previous reports were similar to this research which found that non-pigmented S. *marcescens* was isolated from *C.dactylon* and its nitrogen fixing ability was confirmed. *Klebsiella pneumoniae* was reported as endophytic bacteria in maize[13]. In wheat, Iniquez et al[20] demonstrated and confirmed the nitrogen fixing activity of *K. pneumoniae*. Besides that the production of IAA and related compound by *K. pneumoniae* in culture media supplement with tryptophan was found[17]. The nitrogen fixation, growth promotion and biocontrol activities of *K. pneumoniae* isolates were again confirmed by our work.

Our study clearly showed that the plants growing under severe salinity stress conditions having novel diazotrophs and highly suitable salinity affected areas. The results of the present study also revealed that most of these diazotrophic bacteria were able to produce a variety of plant growth promoting substances such as IAA, GA and biocontrol activity in considerable amounts apart from diazotrophy.

Table 1
Physico-chemical, microbial population and soil biochemical activity of soils obtained from *C.dactylon*

Soil properties	Non-Rhizosphere	Rhizosphere
Physico-chemical properties		
pH	8.56 (± 0.02)	8.29 (± 0.02)
EC (dSm^{-1})	4.8 (± 0.03)	4.7 (± 0.02)
Organic C (mg/kg)	0.36 (± 0.09)	0.42 (± 0.10)
Total N (kg/ha)	176 (± 12)	311 (± 15)
Total P (kg/ha)	8.25 (± 2.15)	17.7 (± 2.79)
Total K (kg/ha)	221 (± 20)	265 (± 22)
Microbial Population		
Bacteria 10^7 cfu g^{-1} of soil	9.0 (±1.15)	12.0 (±0.58)
Diazotrophs 10^4 cfu g^{-1} of soil	5.6 (±0.88)	6.6 (±0.88)
Fungi 10^4 cfu g^{-1} of soil	10.6 (±2.33)	14.3 (±3.18)
Actinobacteria 10^3 cfu g^{-1} of soil	7.0(±1.53)	5.0(±2.52)
Soil biochemical activity		
FDA hydrolysis rate (µg flurescein g^{-1})	9.89 (±0.20)	14.09 (±0.30)
Arginine deaminase activity (µg g^{-1} soil h^{-1})	2.3 (±0.14)	4.4 (±0.17)

Values are mean (± standard error) (n=3)

Table 2
Growth on N-free medium, total nitrogen content and ammonia production of the diazotrophic isolates

S. N.	Isolates	Growth @ 660nm	Total nitrogen mg/g of malate	Ammonia mg/ml
1.	CD1	++	10.3 (±0.12)^{efg}	3.1 (±0.24)^{b}
2.	CD2	+	8.4 (±0.13)^{ghi}	2.7 (±0.10)^{bc}
3.	CD3	+	10.5(±0.45)$^{d-g}$	2.3 (±0.13)^{cde}
4.	CD4	++	12.6 (±0.14)^{bcd}	3.8 (±0.17)^{a}
5.	CD5	+++	9.4 (±0.01)^{fgh}	2.0 (±0.16)^{def}
6.	CD6	++	14.9 (± 0.12)^{a}	2.2 (±0.14)^{cde}
7.	CD7	++	9.1 (±0.35)^{fgh}	2.3 (±0.23)^{cde}
8.	CD8	++	10.4(±0.16)$^{d-g}$	2.3 (±0.14)^{cde}
9.	CD9	++	2.1 (±0.48)^{j}	2.0 (±0.20)^{def}
10.	CD10	+++	2.9 (±0.42)^{j}	1.5 (±0.10)^{fg}
11.	CD11	+	9.5 (±0.16)$^{e-h}$	2.3 (±0.07)^{cde}
12.	CD12	+	14.6 (±0.53)^{ab}	4.3 (±0.10)^{a}
13.	CD13	+	9.4 (±0.10)^{fgh}	4.2 (±0.26)^{a}
14.	CD14	+	1.1 (± 0.01)^{j}	-
15.	CD15	+	2.2 (±0.23)^{j}	-
16.	CD16	++	1.8 (±0.56)^{j}	-
17.	CD17	+	-	-
18.	CD18	+	7.4 (±0.58)^{hi}	-
19.	CD19	++	10.1 (±0.69) ^{abc}	1.3 (±0.08)^{g}
20.	CD20	+++	13.8 (±0.89)^{def}	2.2 (±0.13)^{cde}
21.	CD21	+++	10.9 (±0.98)^{ghi}	2.7 (±0.13)^{bc}
22.	CD22	+	8.4 (±0.78)^{cde}	2.1 (±0.09)$^{c-f}$
23.	CD23	+++	11.8 (±0.42)^{fgh}	2.2 (±0.23)^{cde}
24.	CD24	+	-	-
25.	CD25	+	9.1 (±0.20)^{fgh}	2.5(±0.27)^{cd}
26.	CD26	+	7.2 (±0.84)^{hi}	3.1 (±0.12)^{b}
27.	CD27	++	6.4 (±0.70)^{i}	2.5 (±0.15)^{cd}
28.	Az 204*	++	5.50 (±0.60)	1.8 (±0.13)^{efg}
29.	Control	0.10	0.04	0.93

- No ammonia production & total N content; **Azospirillum lipoferum*;
Values are mean (± standard error) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

Table 3
Nitrogenase activity of diazotrophs obtained from rhizosphere of *C. dactylon*

S. N.	Isolates	Nitrogenase activity (n moles of ethylene mg^{-1} of protein h^{-1})
1.	CD1	-
2.	CD2	96.58(±1.51)^{c}
3.	CD3	-
4.	CD4	131.58(±1.27)^{b}
5.	CD5	95.91(±1.41)^{c}
6.	CD6	174.25(1.89)^{a}
7.	CD7	-
8.	CD8	127.7(±1.40)^{b}
9.	CD9	-
10.	CD10	84.45(±0.98)^{c}
11.	CD11	131.79(±1.46)^{b}
12.	CD11	89.20(±1.43)^{c}
13.	CD12	145.3(±1.35)^{b}
14.	CD13	-
15.	CD19	-
16.	CD20	140.25(±1.95)^{b}

17.	CD21	-
18.	CD22	-
19.	CD23	136.0(±1.23)[b]
20.	CD26	-
21.	CD27	130.57(±1.57)[b]
22.	Az 204*	45.29(±1.03)[d]

**Azospirillum lipoferum*; Values are mean (± standard error) (n=3)
and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

Table 4
PGPR, mineral solubilzation and biocontrol activities of diazotrophic isolates obtained from *C.dactylon*

S. N.	Isolates	[1]Phytohormone production		Mineral solubilzation		[1,2]Siderophore production	[1]HCN production	Antagonistic activity	
		IAA	GA	P	ZN			*P. oryzae*	*R. solani*
1.	CD2	-	-	+	-	-	-	-	-
2.	CD4	12.1(±0.44)[de]	9.8(±0.19)[bc]	+	-	22.35(±0.10)[b]	35.4(±1.24)[b]	+	+
3.	CD5	-	-	-	-	-	-	-	-
4.	CD6	26.7(±0.25)[a]	22.4(±0.23)[d]	+	+	58.44(±0.24)[a]	41.6(±1.19)[b]	+	+
5.	CD8	17.5(±0.52)[b]	21.7(±0.19)[d]	+	+	23.47(±0.11)[b]		+	+
6.	CD10	-	-	-	-	-	-	-	-
7.	CD11	-	-	-	-	-	-	-	-
8.	CD12	12.3(±0.24)[cde]	35.4(±0.10)[a]	+	+	20.98(±0.15)[b]	-	+	+
9.	CD20	24.6(±0.23)[a]	23.7(±0.25)[d]	+	-	60.61(±0.14)[a]	61.8(±1.18)[a]	+	+
10.	CD23	8.4(±0.15)[e]	5.1(±0.04)[e]	+	+	12.47(±0.13)[c]	-	-	-
11.	CD27	16.7(±0.54)[bc]	12.1(±0.11)[c]	+	-	17.94(±0.24)[bc]	30.9(1.09)[b]	-	-
12.	*Pf1	14.9(±0.19)[bcd]	6.1(0.67)[ab]	+	+	20.89(±0.26)[b]	32.8(1.04)[b]	-	-

**Pseudomonas fluorescence*, [1]µg g[1] of protein, [2]Catechol type siderophore;
Values are mean (± standard error) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

Table 5
Effect of diazotrophic isolates on the growth parameters of rice seedlings at 30 Days after inoculation

Treatments	Biomass (g)	Plant height (cm)		Nitrogen content (%)
		Shoot length	Root length	
T1- CD4	0.084 (± 0.013)[b]	16.25 (± 0.92)[c]	8.1 (± 0.35)[b]	1.37 (± 0.13)[d]
T2- CD6	0.098 (± 0.011)[a]	19.56 (± 0.72)[a]	9.3(± 0.23)[a]	1.58 (± 0.23)[b]
T3- CD8	0.065(± 0.016)[d]	14.81(± 0.56)[e]	7.8(± 0.34)[c]	1.46(± 0.45)[c]
T4- CD11	0.087(± 0.041)[b]	13.59(± 0.76)[f]	7.5(± 0.31)[c]	1.34(± 0.24)[d]
T5- CD12	0.078(± 0.081)[c]	15.89(± 0.72)[d]	6.8(± 0.41)[d]	1.23(± 0.23)[e]
T6- CD20	0.097 (± 0.010)[a]	19.00 (± 1.04)[a]	8.3 (± 0.57)[b]	1.88 (± 0.06)[a]
T7- CD23	0.067(± 0.010)[d]	17.34 (± 1.04)[b]	7.4 (± 0.57)[c]	1.37 (± 0.06)[d]
T8- *Az 204	0.094 (± 0.011)[a]	17.04 (± 0.47)[b]	9.3 (± 0.82)[a]	1.52 (± 0.11)[b]
T9- Control	0.050(± 0.003)[e]	10.89 (± 0.59)[g]	6.79 (± 0.62)[e]	0.70 (± 0.07)[f]

**Azospirillum lipoferum*; Values are mean (± standard error) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

Table 6
Molecular characteristics of the isolates

Code of the isolate	Length of the 16srDNA sequence (bp)	Most closely related organism	% of similarity
CD6	1540	*Serratia sp.*	99
CD20	1469	*Klebsiella pneumoniae*	99

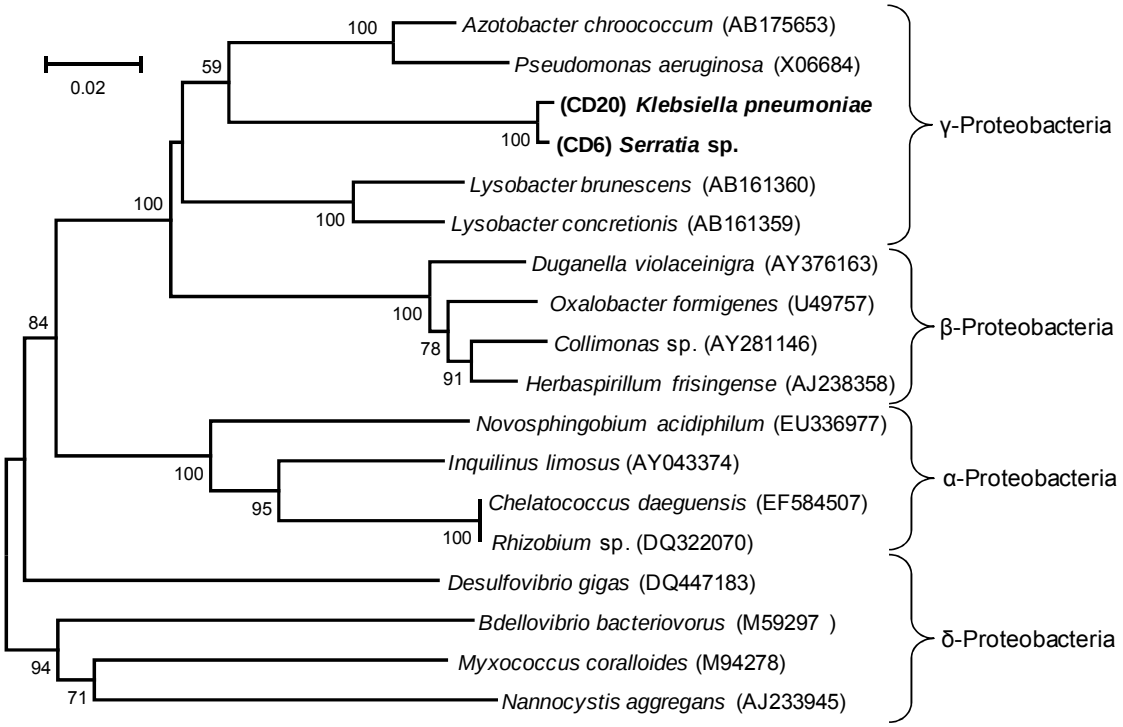

Figure 1: Phylogenetic relationship based on neighbor-joining analysis of 16S rDNA sequence data from the selected isolates

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