नील हरित शैवाल के निवेशन का घान के सूक्ष्म बायोम पर प्रभाव

MICROBIOME OF RICE AS INFLUENCED BY CYANOBACTERIAL INOCULATION

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MICROBIOME OF RICE AS INFLUENCED BY CYANOBACTERIAL INOCULATION

By

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CERTIFICATE

This is to certify that the thesis entitled **“Microbiome of rice as influenced by cyanobacterial inoculation”** submitted to the Faculty of the Post-Graduate School, **Indian Agricultural Research Institute, New Delhi**, in partial fulfillment of **Master of Science in Microbiology**, embodies the results of bonafide research work carried out by **Himani Priya, Roll No. 20313** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged.

Place: New Delhi
Date :

(Radha Prasanna)
Chairperson
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Dedicated

To

Mummy & Papajee

"Whose Love, Blessing, Sacrifice and Perpetual Affection Always Inspired Me to Attain Higher Values of Life and Academic Achievements"

✍...Himani

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1. INTRODUCTION

Rice is the most vital food crop of the developing world and an important staple food for more than 60% of the Indian population. It occupies about 24 per cent of gross cropped area, and contributes 42 percent of total food grain production and 45 percent of total cereal production in India. Rice fields represent unique aqua-terrestrial ecosystems supportive of a tremendous diversity of other plants, fauna, and microorganisms with a wide range of functional capabilities due to nitrogen fixers, nitrifiers, methanogens, methane oxidizers, phosphate-solubilizers, sulfur oxidizers, degraders of pesticides and many others. This diversity is inclusive of Archaea and bacteria that include Cyanobacteria, Proteobacteria and Planctomycetes, as well as the increasing members of endophytes associated with the host plants (Roger and Watanabe 1986; Liesack *et al.* 2000). The complexity and dynamic nature of this ecosystem requires in-depth investigations on the tripartite interactions among plants, microbes and the soil–water environment. This needs to be complemented with studies on the ecological compartmentalization due to diffusion gradients of nutrients and gases such as oxygen which is critical for the growth of many aerobic microorganisms.

The diversity of microorganisms associated with plant roots is enormous; in the order of tens of thousands of species. This complex plant-associated microbial community, also referred to as the second genome of the plant, is crucial for plant health (Berendsen *et al.* 2012). The rhizosphere of many plants is one of the richest microbial systems on Earth (Hirsch and Mauchline 2012). Recent advances in research on plant–microbe interactions revealed that plants are able to shape their rhizosphere microbial communities which were evident from the specific microbial guilds supported by different host plants when grown on the same soil. Plants are also able to recruit protective microorganisms, and enhance certain microbial activities in order to suppress pathogens in the rhizosphere (Berendsen *et al.* 2012). Often, the root-associated microbiota of plants is distinct from those found in the surrounding soils. Hitherto, the endophytic microbiota is poorly described that may have a major contributing role in plant growth, productivity, and carbon sequestration (Rodriguiz *et al.* 2008). The ‘microbiome’ can either benefit or undermine health of rice plants depending upon the nature of interactions. Mutualistic interaction may provide accessible nutrients and phytohormones, which boost up plant growth, or may mitigate phytopathogenic actions or help plants to sustain abiotic stresses (Mendes *et al.* 2011; Firakova *et al.* 2009).

The ‘soil microbial loop’ hypothesis suggests that the cycling of C and N pools between soil and microbial pools has significant implications on plant growth and yields, besides the maintenance of soil fertility (Wardle 2004). Several microorganisms of soils or those associated with roots are difficult to be cultured in the laboratory. Numerous attempts have been made to use the *in situ* hybridization of roots or soil with fluorescently labeled probes and direct microscopy. But, there are always discrepancies between those observed under a microscope and those cultured in the laboratory, more so in their counts. The ‘yet to be cultured’ or members of the “soil rare biosphere” contribute enormously to the soil fertility and plant growth.

The comparison of bacterial communities of different *Arabidopsis* genotypes using 454 sequencing of 16S rDNA amplicons at different growth stages and different soil types provided valuable information (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012) regarding the preferential colonization by members belonging to Proteobacteria, Bacteroidetes and Actinobacteria, although some researchers earlier reported a bias for Betaproteobacteria. Evaluation of the bacterial communities under the controlled conditions even suggested a link between the metabolically active cells and the cell wall features of the host plant and those soil bacteria selected for the colonization of host plant (Bulgarelli *et al.* 2012). Earlier reports from IARI suggest that cyanobacteria can colonize the roots of several crops, including rice, wheat, tomato, cotton and elicit defense responses in their hosts (Karthikeyan *et al.* 2009; Prasanna *et al.* 2009 a,b; 2011, 2013 a,b,c).

Cyanobacteria are well adapted to diverse environmental milieu and considered the primary colonizers in several inhospitable habitats. They are also of agronomic importance and are often employed as ‘bioinoculant’ for enhancing crop yields, especially in rice, besides improving fertility and structure of soils (Venkataraman, 1972; Kaushik 2004, Nayak *et al.* 2004, Dhar *et al.* 2007*).* Cyanobacteria can sequester carbon, add organic matter, synthesize and liberate amino acids, vitamins and auxins, reduce oxidizable content of soil organic matter, provide oxygen to the submerged rhizosphere, ameliorate salinity, buffer the soil pH, solubilize phosphates and even increase the fertilizer use by the crop plants (Roger *et al.* 1993; Mandal *et al.*,1998; Kaushik 2004). Nevertheless, information is very scanty on the changes in the structure and function of soil or plant microbial communities due to cyanobacterial inoculation of rice plants.

Information on the microbiome of rice plants is necessary, especially in relation to cyanobacterial inoculation since cyanobacteria are also a major discernible component of the rice field biota, and play a significant role in the sustenance of soil fertility. A number of bacteria, especially the Enterobacterial genera such as *Enterobacteria, Serratia, Pseudomonas, Erwinia, Herbaspirillum, Gluconoacetobacter* are known to proliferate in rice rhizosphere and play a significant role in soil and crop productivity (Malik 2002). The cyanobacteria are mainly explored to-date as above-ground photosynthetic flora despite their capabilities to function as mixotrophs. Cyanobacteria are a remarkable group of prokaryotes, which can exist independently, symbiotically or facultatively with a diverse range of members of plant kingdom like Gymnosperms, Pteridophytes and Bryophytes (Rai and Bergman 2002). However, their association with crop plants are less explored (Nilsson *et al.* 2005).

No plant can exist without microorganisms and a completely microbe-free plant is an improbable exception, rather than the biologically relevant rule (Partida-Martinez and Hiel 2011). Considering the above information from the scientific literature, it was proposed to investigate the rice plant microbiome (microbial communities in the soil, rhizosphere, roots, shoot and leaves) in the presence or absence of cyanobacterial inoculation under controlled conditions.

The research approaches included both culturable and culture-independent methods (phospholipid fatty acid; PLFA and denaturing gradient gel electrophoresis; DGGE profiling), complemented by soil or plant analyses and microscopic investigations. There is a strong need to identify the major taxonomic groups of microorganisms associated with rice plants, and their interactions due to the cyanobacterial inoculation. This would improve our understanding of the plant – microbe interactions and the ‘core microbiome’ of rice plants for the development and effective utilization of new microbial inoculants, and gain improved nutrient uptake complemented with enhanced soil fertility and crop productivity.

Objectives

- To evaluate the effect of cyanobacterial inoculation on the culturable nitrogen-fixers and P solubilizers in the rice microbiome
- To monitor changes in the microbial community at specific growth stages of rice crop

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3. BACKGROUND

Rice (*Oryza sativa* L.) is one of the world's oldest and most important crop species, having been domesticated about 8,000–9,000 years ago. Broadly speaking, rice is grown in more than 100 countries, with a total harvested area of about 153 million ha, and production of more than 600 million tons annually. Yields range from less than 1 t ha^{-1} under very poor rainfed conditions to 10t ha^{-1}in intensive temperate irrigated conditions (Barker et al. 1985; Zeigler and Barclay 2008). The consumption of rice exceeds100 kg per capita annually in many Asian countries, providing 20% of the direct human calorie intake. Asia accounts for over 90% of the world's production of rice, where the poorest of the poor spend up to 50% of their total income on rice alone. It is estimated that in Asia, the rice consumption by the year 2025, over the base year 1995, will increase by more than 51 percent. The current demand of 524 million tonnes is expected to increase to over 700 million tones, which would necessitate heavy inputs of fertilizers and chemicals, besides taking over land, currently under forest cover. This in turn would lead to further increase in environmental pollution and soil health, which can be alleviated through the use of biofertilizers or biopesticides in an environment-friendly manner.

It is now well known that the diversity of microbes associated with plant roots is enormous; in the order of tens of thousands of species. This complex plant-associated microbial community, also referred to as the second genome of the plant, is crucial for plant health (Berendsen et al. 2012). Recent advances in research on plant–microbe interactions have revealed that plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host have characteristic microbial communities when grown on the same soil (Aira et al. 2010; Boyle and Patriquin 1980; Kuske et al. 2002). This can be a prelude to improving the efficiency of microbial inoculants and promoting environment-friendly and green agriculture.

Microbiology of rice soils

Rice fields are important and unique in ecosystems because of the presence of diverse, biologically important microorganisms. These fields represent unique aqua-terrestrial ecosystems in which the tremendous diversity of soil microbes, soil fauna and plants – ranging in function from nitrogen fixers, nitrifiers, methanogens, methane oxidizers, phosphate-dissolving microbes, sulfur oxidizers to the catabolizers of pesticides is observed. Tremendous differences were also observed among microbial populations – both qualitative and quantitative, in tropical and temperate soils (Sethunathan *et al.* 1982; Ladha 1986), as well as the composition of microflora in wetland and upland rice soil. An upland rice soil comprises essentially an oxic environment, except for rare localized anoxic pockets (pores of soil particles or in soil aggregates). Also, some commonly observed microorganisms in submerged soil include genera of *Spirillum, Bacillus, Pseudomonas, Mycobacterium* and *Arthrobacter,* besides nonsulfur and sulfur photosynthetic bacteria (Lakshmi Kumart *et al.* 1976). However, the rhizosphere effect (R: S, ratio of microorganisms in the rhizosphere to microorganisms in the non-rhizosphere) was observed to be lower for oxic and facultative microorganisms under submerged conditions compared with upland conditions, and greatest at the transplantation stage (Kimura *et al.* 1979).

Aerobic vs anaerobic microbial diversity in rice field

Paddy field may represent the most important agricultural ecosystems in Asia since rice is the main source for food supply. Rice field is flooded for most of the period of rice cultivation and is left under drained conditions during the off-crop season. The paddy field ecosystem, therefore, consists of diverse habitats for microorganisms in time and space, such as aerobic/anaerobic soil conditions, floodwater, rice roots, rice straw stubble and composted materials for growth and development of micro-organisms (Raipuria *et al.* 2013; Reche and Fiuza 2005). In addition, gradients from stagnant to percolating water provide environments with different oxygen levels. These habitats are abiotically different microenvironments that could exhibit biologically distinct properties. Such heterogeneity of the habitats should influence the structure and diversity of microbial communities in the paddy field ecosystem as a whole and may support various microbiological processes occurring in paddy fields, most of which are agronomically and biogeochemically important (Dommergues *et al.* 1973; Keyeo *et al.* 2011; Kimura 2000; Kirk 2004). The rhizosphere, which represents the soil area under the direct influence of the roots, serves as a dynamic circumjacent site with intense microbial activity, and represents a favorable aerobic–anaerobic interface suitable for diverse groups of micro-organisms. From many studies it is reported that there is a clear change in the composition of microbial community under different growth conditions such as flooded and non-flooded conditions. Bai et al. (2000) reported a higher ratio of Gram-positive bacteria to Gram-negative bacteria, ranging between 0.90 and 0.94 for flooded compared with non-flooded soils (0.78). Genera commonly observed in submerged soil include *Bacillus*, *Pseudomonas*, *Mycobacterium* and *Arthrobacter*, in addition to nonsulfur and sulfur photosynthetic bacteria. The most abundant gram positive genera present in rice fields are *Bacillus*, *Corynebacterium*, *Staphylococcus* and *Lactobacillus* homogeneously distributed in all studied rice crop areas. Anaerobic microorganisms such as fermentative bacteria and methanogenic archaea predominate within the microbial community, producing methane as the final product of anaerobic degradation of organic matter. Both methane- and ammonia-oxidizing bacteria can act as a sink for methane in rice soils, due to the homology of the key enzymes methane monooxygenase and ammonia monooxygenase. Sakai et al. (2007) isolated a methanogen (strain SANAE) belonging to RC-I from Japanese rice by co-culture with the propionate-oxidizing and hydrogen producing syntroph, *Syntrophobacter fumaroxidans*. Most interestingly, root colonization with RC-I methanogens was denser than with Methanomicrobiales, so that the plant colonized with RC-I produced and emitted relatively more methane (Conrad *et al*. 2008).

Phototrophic v/s heterotrophic microbial diversity in rice field

Aerobic anoxygenic phototrophic bacteria (AAnPB) are recognized as an important group driving the global carbon cycling. AAnPB are found in a wide variety of environments (Yurkov 2006) and thought to be of ecological significance (Kolber *et al.* 2001; Jiao *et al.* 2007). Flooding management makes paddy field a unique aquatic system because ofthe co-occurrence of both anoxic and oxic niches. For instance, during rice growing period, soil is mainly characterized as an anaerobic system, while there are oxic zones such as rhizospheric There exists a diverse AAnPB community with large population size residing in paddy surface soil. Although the depth of photic paddy soil is usually between 0.2 and 2 cm (Roger 1996), the unilluminated zone of surface paddy soil that is ploughed as a result of agricultural practices will become the illuminated zone following plowing the next year. Thus, the surface soil at a depth of 0–5 cm is presumably suitable for the investigation of phototrophs. In fact, the investigations of the diversities of algae (Fujita and Nakahara 2006) and cyanobacteria (Song et al. 2005) are also conducted at such depth in paddy surface soil. AAnPB are found to be widespread in almost all extant ecosystems (Yurkov 2006). Culture-independent analysis reported that AAnPB could occur in geographically distinct ecosystems, while certain unique AAnPB could be particularly favoured in paddy soil. The *pufM* genes in paddy soil within thephyla of *Alpha*- and *Gammaproteobacteria* were similar to those of AAnPB isolated from ocean such as *Rubrivivax gelatinosus* and *Rhodobacter blasticus* and from lake water such as *Acidiphiliumcryptum* (Hu et al. 2006; Waidner and Kirchman 2008). However, the deeply branching cluster of *pufM* genes related to *Methylobacterium* sp. and *Bradyrhizobium* sp. was detected only inpaddy soil, which might extend the functional ecotypes ofAAnPB. Most diverse photosynthetic bacteria, purple nonsulfur bacteria (PNSB) are adaptable organisms known to occur in the water column of rice fields.

Cyanobacteria or blue green algae as they are commonly called are an important inhabitant of rice paddies and well documented for their role is sustaining the fertility of these soils. Studies on cyanobacterial diversity of rice fields belonging to diverse agro-ecologies of India have shown the predominance of certain genera, particularly *Nostoc, Anabaena* and *Phormidium,* irrespective of chemical/biofertilizer treatments and stage of crop growth (Singh and Bisoyi 1989; Ghosh and Saha 1997; Nayak *et al*. 2001, 2004; Nayak and Prasanna 2007). Investigations undertaken to characterize the abundance, genus-level diversity and metabolic capabilities of cyanobacteria isolated from the rice rhizosphere of different rice varieties belonging to diverse soil types, revealed that the genera *Nostoc* and *Anabaena* comprised 80% of the rhizosphere isolates (Nayak *et al.* 2009; Prasanna *et al.* 2009 a,b). Cyanobacteria are known to liberate a wide array of extracellular substances, such as plant growth regulators, vitamins, amino acids and sugars, which have direct or indirect impact on plant growth (Misra and Kaushik 1989; Prasanna *et al.* 2008) and surrounding microflora.

Oligotrophic v/s eutrophic organisms

Rice fields represent one of the best-studied model systems in soil microbial ecology, comprising a rich diversity of plant growth-promoting bacteria (PGPB) such as N2-fixers, phosphate-solubilizing bacteria (PSB), fluorescent bacteria and endophytes (Cong *et al.* 2009; Islam *et al.* 2010). The significance of the microbially active rhizosphere to the nutrition and growth of rice plants, especially in relation to serving as an effective medium for nitrogen fixation and detoxification of pesticides/agrochemicals has been the subject of intensive global research (Sethunathan *et al.* 1982; Lu *et al.* 2004; Das and Baruah 2008).A large number of micro-organisms live and survive with difficulty in the soil environment because of unavailability and low rate of supply of nutrients. Therefore**,** it may be advantageous for soil microorganisms to have the capability of growing in low nutrient conditions. Micro-organisms possessing this capability are called as oligotrophs or diluted nutrient bacteria (DNB) (Hirsch et al. 1979; Kuznetsov *et al*. 1979; Ohta and Hattori 1983). In rice fields, oligotrophic bacteria are present predominantly on organic debris throughout the period of rice cultivation, besides dominating in rice roots and other plant parts. It has been also reported that, DNB were dominated under anaerobic conditions. Flooded conditions are favorable for the build of oligotrophic bacteria especially those capable of growing under anaerobic conditions. The populations of anaerobic oligotrophic bacteria are also more than the population of aerobic oligotrophic bacteria in the roots of rice. However; the numbers of NB bacteria both aerobic and anaerobic were almost identical (Sridevi *et al.* 2013). A diverse array of non-destructive methods has been employed for examining root dynamics through the use of below-ground rhizosphere images such as the use of mini rhizotron (Hendrick and Pregitzer 1992) or rhizobox (Watt *et al.* 2006). Identification of acetate-assimilating microorganisms (eutrophic micro-organism) under methanogenic conditions in anoxic rice field soil was done by Comparative Stable Isotope Probing of RNA, which revealed that *Geobacter* spp. and

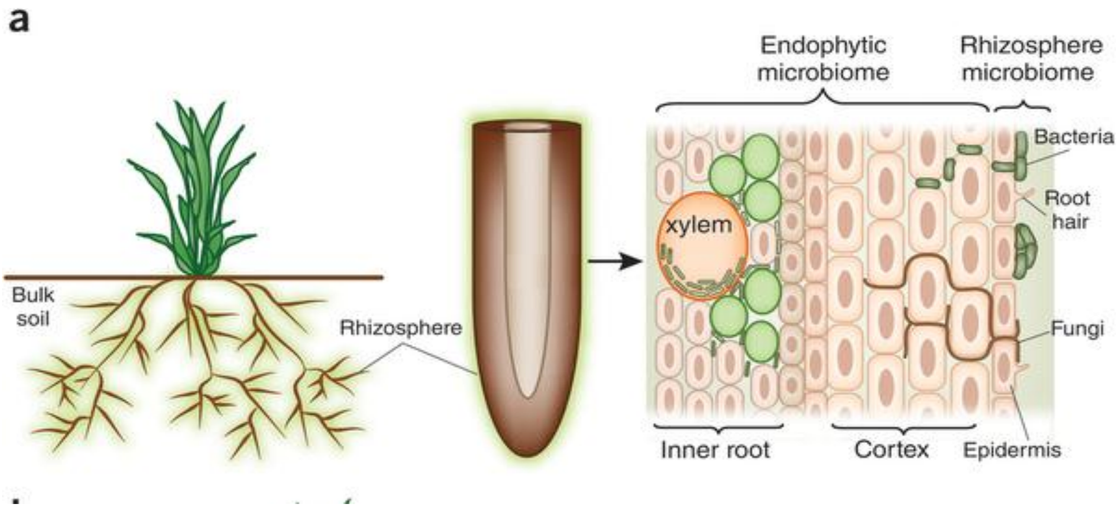
Anaeromyxobacter spp. assimilated acetate during the methanogenic phase of a rice field soil.

Rice microbiome

The natural interactions between plant roots and their rhizospheric microbiome are vital to plant fitness, modulating both growth promotion and disease suppression, which may be the result of a complex interplay of mechanical and chemical signals (Jayaraman *et al.* 2014). Naturally occurring root-associated rhizospheric bacteria were isolated from California field grown rice plants (M-104), eleven of which were taxonomically identified by16S rRNA gene sequencing and fatty acid methyl ester (FAME) analysis (Spence *et al.* 2014). The rice plant represents a habitat for diverse micro-organisms, those that colonize the aerial parts (phyllosphere), the root surface (rhizoplane), as well as the zone around the root (rhizosphere) (Knief *et al.* 2012). Most studies related to identification of organisms in the phyllosphere have mainly focused on bacteria and, to a lesser extent, fungi (Vorholt 2012). Potential beneficial interactions of phyllosphere bacteria with rice plants, such as plant growth promotion by bacterial nitrogen fixation or plant hormone production have been studied (Knief *et al*. 2012).

Rice rhizosphere

A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere; bacteria are the most abundant among them. The rhizosphere represents one of the most intrinsically complex ecosystems on earth with almost every root on the planet expected to have a chemically, physically and biologically unique signature (Jones and Hinsinger 2008). In the current scenario, rhizosphere research is addressing the issue of spatial complexity in several ways. One of the key challenges faced by rhizosphere ecologists is the translation of fundamental research into practical applications in cropping systems. In this context, considerable advances have been made in understanding the interactions among rice plants, soil biogeography, soil micro-/macro-fauna and microorganisms (Heur and Smalla 1997; Hori *et al.* 2007; Watt *et al*. 2006; Xie *et al.* 2003).



Lower and higher plants have endophytes that can be found intracellularly, such as the endosymbiotic rhizobia, either as obligate or in the facultative association (Harley, 1983; Hinton and Bacon, 1985). In many cases, plant-endophytic bacteria do not damage the host organism (Khush and Bennett 1992; Misaghi and Donndelinger, 1990; Quispel, 1992); sometimes, endophytic bacteria are even potential sources of resistance against pathogenic agents, such as fungi (Benhamou et al. 1996). The bacteria and fungi growing in the rhizosphere, rhizoplane and phyllosphere were isolated from rice growing regions of Kenya and strains with promising physiological characteristics, including P-solubilization, N-fixation and IAA production were recorded (Mwajita *et al.* 2013).

The rich microbial activity in the rhizosphere aids in several biological and ecological processes important for plant health. Culture-independent molecular techniques based study (16S rRNA clone library generation along with RFLP, sequencing and phylogenetic analysis) was performed to investigate the bacterial diversity associated with the rice rhizosphere bacterial communities from a paddy field ecosystem in Kerala. Sequence analysis of 16S rRNA clones indicated a high diversity in the rhizosphere bacterial community with the majority of microbes being closely related to the *Proteobacteria*. Only a small fraction of the 16S rRNA sequences were highly similar to rRNA sequences from *Acidobacteria, Firmicutes, Bacteriodetes* groups (Arjun *et al*. 2011). Since rhizosphere-associated microbes possess diverse metabolic capabilities and play a crucial role in plant health, knowledge on their community structure is imperative for the proper understanding of their individual roles and metagenomics holds the promise to reveal several important questions regarding the unculturable fraction of the rhizosphere community. The diversity and activity of microbial communities in the rhizosphere of irrigated rice soils influence the soil fertility and nutrient use efficiency (watanabe and Cabrera 1979; Ushio *et al*. 2014).

Lucas et al. (2013, 2014) isolated 900 isolates from the rice rhizosphere; among them, only 38% were positive for at least one of the biochemical activities of significance as putative PGPR. Shrivastava *et al.* (2014) isolated some rhizospheric microorganisms which were promoting growth of rice grown in 10 zones of district Durg of Chhattisgarh state and exhibited siderophore production, indole-3-acetic acid production and phosphate solubilization.

Jin *et al. (*2014) using the 16S rRNA gene clone library technique, the bacterial community structure and diversity of the rhizosphere and endophytic bacteria in *Stellera chamaejasme* were compared and clarified for the first time. Grouping of sequences obtained showed that the members of the *Proteobacteria* (43.2%) *Firmicutes*(36.5%) and *Actinobacteria* (14.1%) were dominant in both the rhizosphere and endophytes. other groups of micro-organisms mainly Bacteroidetes (2.1%), Chloroflexi (1.9%) and Cyanobacteria (1.7%) were consistently found in lower numbers. In this study, the community analysis of the rhizosphere vs endophytes and organs (leaf, stem and root) indicated that more varied bacteria inhabited the rhizosphere compared to the organs of plants.

Rice endophytes

Although the importance of plant-associated microorganisms for plant growth and health is well documented, the role of seed-associated microorganisms, especially seed endophytic bacteria, is still underestimated (Kloepper and Ryu 2006). Such associations could be beneficial for germination and seedling establishment, as seed endophytic bacteria are already present in these very early plant growth stages (Kovtunovych *et al* 1999). Moreover, bacteria with beneficial characteristics can be selected by the plants and could be transferred via the seed to benefit the next generation (Truyens *et al*.

2014). In a study of the rhizoplane of rice plantlets of *Oryza sativa*, varieties-Morelos A-88 and Apatzingan isolated two bacterial strains: *Corynebacterium avescens* and *Bacillus pumilus*. Scanning electron microscopy, endophytic bacteria were frequently identified at the base of secondary roots, between the epidermis and the mucilaginous layer. Comparison of the length of inoculated or nalidixic acid-treated plantlets, with non-inoculated plantlets revealed a significant enhancement of the growth of the shoots at 15 days of culture in plantlets colonized exclusively by endophytes. *A. brasilense* seems to be excluded from the rhizoplane by the endophytic bacteria, suggesting that endophytes compete with *Azospirillum*, and also that *A. brasilense* inhibits growth of rice. This investigation on rice plant microbiome indicates that endophytic bacteria participate actively in the growth and development of rice plants (Baciio-Jimeanez *et al*. 2001; Huang 1986).

Yanni *et al.* (1997) documented a beneficial natural endophytic association of rhizobia and rice grown in rotation with clover, using a legume trap host to specifically enumerate and isolate endophytic rhizobia from rice roots. Mano and Morisaki (2008) have provided a very comprehensive survey of the diversity of cultivable and uncultivable bacteria from various organs of the rice host plant. They observed more population divergence in rice roots than in the seeds and leaves. Using a set of bacterial PCR primers for selective amplification of bacterial 16S rDNA sequences from root tissues, 52 OTUs (operational taxonomic units) were identified (Sun *et al.* 2007). Sequence analyses revealed diverse phyla of bacteria, comprising alpha, beta, gamma, delta, and epsilon subclasses of the Proteobacteria, the Cytophaga/Flexibacter/Bacteroides (CFB) phylum, low (GþC) gram-positive bacteria, the Deinococcus/Thermus group, Acidobacteria, and Archaea. The endophytic bacterial flora in different parts of the rice plant is diverse, with *Pantoea* being isolated from seeds, *Methylobacterium* from the shoots, *Azospirillum* and *Herbaspirillum* from the stems and roots, and *Burkholderia* and *Rhizobium* from the roots. Among the large and diverse range of N_2 fixing heterotrophic bacteria isolated from surface-sterilized rice plants, only a few – notably *Alcaligenes faecalis* (later identified as *Pseudomonas stutzeri*A16), *Azoarcus* spp., *Herbaspirillum* spp., *Rhizobium* spp., and *Serratia* *marcescens* IR BG 500 – have been confirmed to be genuine endophytes of rice (Hurek *et al.* 1997; James 2000), as they can only be isolated from host tissues and do not survive well in soil.

Rice phyllosphere

Our knowledge of the microbiology of the phyllosphere, or the aerial parts of plants, has historically lagged behind our knowledge of the microbiology of the rhizosphere, or the below-ground habitat of plants, particularly with respect to fundamental questions such as which microorganisms are present and what they do there. In recent years, however, this has begun to change. The leaves of plants are inhabited by a diverse community of microorganisms. These leaf inhabitants influence their hosts with respect to growth and resistance to abiotic and biotic stresses. The microbial communities of the phyllosphere are diverse, supporting numerous genera of bacteria, filamentous fungi, yeasts, algae and in some situations protozoans and nematodes (Morris *et al.* 2002; Lindow and Brandl 2003). Bacteria are most numerous and diverse on leaves with culturable counts ranging between10^2 to10^{12} cells per gram of leaf (Thompson *et al.* 1993; Inacio *et al.* 2003). α- and ϒ- proteobacteria are generally the dominant bacterial inhabitants of phyllosphere, with Bacteroidetes being usually important. B- Proteobacteria and Firmicutes can also form a large part of the bacterial community in some situations, with Acidobacteria, Actinobacteria and cyanobacteria occurring infrequently (Kadivar and Stapleton 2006; Idris *et al.* 2004)

Significance of cyanobacteria in rice soil

Cyanobacteria are a unique group of organisms, exhibiting cell structure similar to bacteria, but employ plant-like photosynthesis, and several forms exhibit independence for C and N nutrition. They play diverse roles in the environment, as nutrient supplements (biofertilizers) and soil compaction agents in agriculture, besides having tremendous ecological significance as carbon sequestering and bio-remediating agents (Venkataraman 1972; Prasanna *et al*. 2008a; Prasanna *et al.* 2009b; 2010a; Gupta *et al.* 2013). The role of cyanobacteria as biofertilizers has undergone a major change in recent years, with their utility as not only diazotrophs, but also as plant growth promoting inoculants in a diverse range of crops (Karthikeyan *et al.* 2007; Nain *et al.* 2010; Manjunath et al. 2011).

In recent years, cyanobacteria are also being explored as sources of bioactive molecules having pharmaceutical and agricultural significance (Jaiswal *et al.* 2008; Prasanna *et al*. 2008a, b; Prasanna *et al.* 2009 a,b, 2010b; Manjunath *et al*. 2010). Such bioactive compounds are known to be important determinants of allelopathic activity in water and soil. With the increasing concern to health and environment due to fungicides and resistance development in target pathogens, it has become necessary to intensify research towards the development of novel, more effective and sustainable fungal disease control solutions. The excretion of hydrolytic enzymes is known to be a common trait of plant pathogens/symbionts, which promotes a closer association with plant roots/target organisms and improve the stability of such associations. Prasanna et al. (2008b, 2009a) and Gupta *et al. (*2010; 2011) revealed for the first time, the activity and homologues of hydrolytic enzymes in several *Anabaena* strains and their correlation with fungicidal activity. Studies were undertaken to characterize the abundance, genera-wise diversity and metabolic capabilities of cyanobacteria isolated from the rice rhizosphere, for the first time, revealed that the genera *Nostoc* and *Anabaena* comprised 80% of the rhizosphere isolates (Prasanna *et al.* 2009b). Rhizosphere samples from rice cultivars grown at Aduthurai exhibited low generic richness i.e. only two genera *Anabaena* and *Nostoc* were observed (Prasanna and Nayak 2007; Prasanna *et al.* 2009b). As rhizosphere isolates can be better competitors, due to their direct linkage with roots, therefore, their inclusion in biofertilizer/biocontrol consortia can improve the effectiveness of these inoculants. The rhizosphere dynamics and plant growth promoting ability of a set of inoculated strains in rice crop revealed the persistence of strains on roots upto harvest stage, entry into roots (Karthikeyan *et al.* 2009) and enhancement in plant growth and yield attributes, besides improved soil fertility/microbiological parameters and plant growth parameters (plant height, dry weight, grain yields) carbon (Prasanna *et al.* 2009a). Field evaluation of promising cyanobacterial and bacterial strains revealed the promise of combinations of cyanobacteria-bacteria in enhancing crop biomass and yields. The penetration and presence of cyanobacterial cells and short filaments in the root cortical region have been revealed by electron microscopic investigations (Jaiswal *et al.* 2008; Karthikeyan *et al.* 2009), which further emphasized the potential of these isolates for developing effective associations with roots. The general but inaccurate belief that all cyanobacteria are obligate photoautotrophs has been, perhaps, the major reason for the paucity of information available on the diverse roles of these micro-organisms in the rhizosphere.

Quantitative and qualitative distribution of cyanobacterial genera in rice fields has shown the preponderance of *Anabaena* and *Nostoc*, and the role of pH in the distribution and number of cyanobacterial species (Prasanna and Nayak, 2007; Nayak *et al.* 2001). It is also well known that cyanobacteria play a critical role in the sustenance of fertility of this ecosystem and bring about an improvement in yield of rice (ranging from 5 to 25%), and indirect or direct beneficial changes in the physical, chemical and biological properties of soil and soil-water interface in rice fields, which are of agronomic importance (Kaushik 1998; Nayak et al. 2001,2004; Venkataraman 1981) and a variety of other factors, such as mitigation of methane emissions, besides nitrogen fixation (Prasanna et al. 2002). These include enhancement in soil organic carbon, nitrogen and phosphorus, besides improving soil structure and quality and production of growth promoting substances (Misra and Kaushik 1989; Kaushik 1998). Karthikeyan *et al.* 2007, 2009) demonstrated that cyanobacteria excrete IAA, amino acids and other growth promoting compounds into their immediate environment, which in turn can stimulate the growth of microbial populations in soil, and demonstrated the agronomic potential of these strains, providing the support as ideal candidates for development of inoculants for wheat crop.

Most of the work related to cyanobacterial biofertilizers has been in relation to rice crop, and few reports are available on epiphytic growth of *Gleotrichia pisum* on the aquatic roots / stem of deep water rice (Barraquio *et al.* 1997; Rother *et al.* 1988; Whitton *et al. 1988*) and cyanobacteria attached to the lower epidermis of *Lemna* plants, in rice fields/ associated ponds. At present, scientists are interested in creating artificial symbiosis between higher plants and nitrogen-fixing microorganisms with a view to introduce nitrogen fixing ability in the plants and develop beneficial mutualistic associations (Gusev *et al*. 1986; Rai 1990; Rai *et al.* 2000; Sofi and Wani 2007). Novel associations between *Rhizobium* – rice (Al- Mallah *et al. 1989*) and *Anabaena*-Tobacco have also been reported. Nilsson *et al.* (2002) tested the efficiency of numerous symbiotic cyanobacterial isolates with rice and found that under laboratory conditions, a number of them were successful in forming artificial associations. Interesting associations of selected cyanobacterial strains with wheat seedlings and rice seedlings (Akojijam *et al.* 2010; Babu *et al.* 2014; Gantar *et al.* 1991) have been documented.

A number of compilations on cyanobacteria from different soils of India exist, but emphasis has been solely with respect to their nitrogen fixing potential and limited published work exists on the production of bioactive compounds and their interactions with crop plants. At IARI, New Delhi, which has been the starting point for the "*Algalisation*" technology in India, i.e. inoculation of algae as biofertilizers, the role of cyanobacteria in rice fields, research has been undertaken on their geographic and regional distribution (De, 1939; Prasanna, *et al.* 2007; Venkataraman 1981; Whitton 2000). Cyanobacteria are known to inhabit a wide range of habitats, including plant surfaces (Whitton *et al.* 1988; Mano and Morisaki 2008), but information on cyanobacteria in relation to microbiome of plants is still in its infancy.

3.1 Experimental cyanobacterium: *Calothrix elenkinii*

The axenic culture of *Calothrix elenkinii* (RPC1) was obtained from the Germplasm Collections of the Division of Microbiology, IARI, New Delhi. The purity of culture was examined by microscopic observations at regular intervals during this investigation.

3.1.1. Growth and maintenance of *Calothrix elenkinii*

The strain was grown and maintained using BG-11(-N) medium (Stanier et al. 1971) with suitable pH, at 27±2°C under light: dark cycles (16:8 h).The intensity of white light used was 50-55 µmol photons $m^{-2}s^{-2}$. Sub-culturing was at done at a 3 week intervals.

3.1.2. Composition of medium used

The BG-11(-N) medium was prepared using the following ingredients (g L^{-1}) in distilled water:

K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA	0.001
Na_2CO_3	0.02
Trace metal mix	1 ml

The trace metal mixture contained the following constituents (g L^{-1}) in distilled water:

H_3BO_4	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

The pH of the medium was maintained within the range of 7.3-7.5 for optimal growth of culture.

3.2. Evaluation of growth, morphological and physiological attributes of *Calothrix elenkinii*

3.2.1. Chlorophyll estimation of cyanobacterial culture (Mackinney 1941)

Chlorophyll concentration is used as an index of cyanobacterial growth and measured spectrophotometrically using the hot extraction method. The cyanobacterial culture suspension (10 mL) was harvested by centrifugation at 5000 rpm for 10 min. and methanol (10 mL, 95%) was added to the pellet. The level of methanol was marked on outside of each tube. The contents in all the tubes were swirled thoroughly and placed in a water bath at 60°C for 30 min. Later, the contents were cooled down to room temperature. The evaporation loss, if any, was made up with methanol, and the contents of all the tubes werethen centrifuged at 5000 rpm for 10 min. The pellet was discarded and the optical density (O. D.) was measured at 650 and 665 nm against 95% methanol as blank in a Specord 200 PLUS (Analytikjena, Germany). The amount of chlorophyll 'a' was calculated using following equation:

Chlorophyll 'a' (μg mL^{-1}) = $2.55 \times 10^{-2} \times (\text{O. D.}_{650}) + 0.4 \times 10^{-2} \times (\text{O. D.}_{665})$

3.2.2. Indole acetic acid (IAA) production (Gordon and Weber 1951)

The concentrations of IAA in the culture pellet and filtrate from cultures as determined spectrophotometrically by the method outlined by Gordon and Weber (1951) using IAA as standard prepared in 50% ethanol. To theculture filtrate (2 mL), *o*-phosphoric acid (2-3 drops) was added to acidify the solution. Freshly prepared perchloric acid reagent (4 mL, $FeCl_3$ (1 mL) in 50 mL of 35% $HClO_4$) was added to the acidified solution and incubated at room temperature for 25 min. The intensity of pink colour was measured at 530 nm and the concentrations were interpolated from the standard curve of IAA and expressed as μg mL^{-1}. The concentrations of IAA were estimated at four different growth stages.

3.2.3. Total soluble proteins (Lowry et al. 1951)

The amount of soluble proteins in the culture pellet and filtrate of cultures weredetermined spectrophotometrically according to Lowry et al. (1951) with Bovine serum albumin as standard. To 0.5 mL of the sample, 1 N NaOH (0.5 mL) was added and incubated in boiling water bath for 10 min. After cooling 2.5 mL of Solution 2 (50 mL of 5% Na_2CO_3 mixed with 2 mL of 0.5% $CuSO_4.5H_2O$ in 1% Sodium potassium tartrate) was added and the contents were incubated for 10 min at room temperature. The Folin-Ciocalteu Reagent (0.5 mL, 1N) was added later and the contents were further incubated at room temperature for 15 min. The blue colour developed was recorded spectrophotometrically at 650 nm.

3.2.4. Assays for hydrolytic enzyme activities

The cell free culture filtrates of cyanobacterial strain were analysed for activities of hydrolytic enzymes such as chitosanase (EC 3.2.1.132), β-1, 3-glucanase (EC 3.2.1.39) and β-1, 3-glucanase (endoglucanase) at different periods of culture growth. The activity of a hydrolytic enzyme was expressed as standard International Unit (IU).

3.2.4.1. Chitosanase activity (Ohtakara 1988)

The cell free culture filtrates of *Calothrix elenkinii*were analyzed for chitosanase (EC 3.2.1.132) activity using glycol chitosan as assay substrate by dinitrosalicylic acid method. The standard assay was prepared by mixing of 0.5 mL of sample(extracellular filtrate) and 0.1 mL of glycol chitosan (1% in 0.1N sodium phosphate buffer, pH 7.5) and incubated for 1 h at 37°C to allow for completion of hydrolysis.Acetyl acetone reagent (0.5 mL, prepared as 1 mL acetyl acetone in 50 mL of 0.5 M $Na_2CO_3$and used within one hour) was added, and the resulting mixture was boiled for 15 min.Ethanol (2.5 mL) and Ehrlich's reagent (0.5 mL, 0.8 g of p-di-methyl amino benzaldehyde in 30 mL of ethanol added to 30 mL of concentrated hydrochloric acid) were then added. The tubes were incubated for 65-70°C for 10 min in water bath followed by centrifugation to isolate insoluble chitosan. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm. One unit of enzyme activity was defined as µmoles of reducing sugars (expressed as glucosamine equivalents) released $min^{-1}mL^{-1}$ culture filtrate under assay conditions.

3.2.4.2. β- 1, 3-glucanase activity (Ohtakara 1988)

Preparation of Dinitro salicylic acid reagent (DNSA): This reagent was prepared freshly every time and kept in dark bottles.

Solution A: To 1% NaOH solution, the following reagents were added and the volume was made upto1000 mL:

DNSA (3,5-Dinitrosalicylic acid)	10g
Phenol crystals	2g
Na-K tartarate	200mg

Solution B: 5% Sodium sulphite

Working solution of DNSA: 99 mL of solution A was added to 1 mL of solution B.

The β- 1, 3-glucanase activity was spectrophotometrically determined by measuring reducing sugars released by hydrolysis of laminarin, which was used as substrate. The sample of culture filtrate (0.5 mL) was taken in the tubes and then sodium citrate buffer (1 mL, pH 5.1) and laminarin (125 µl, prepared at a concentration of 1.6 mg mL^{-1}) were added. The tubes were incubated at 30˚C for one and half hours and kept in boiling water bath for 4-5 min. TheDNSA reagent (3 mL) was added and tubes were incubated at 100˚C for 15-16 min. The activity of β- 1, 3-glucanase (EC 3.2.1.39) was determined spectrophotometrically at 530 nm, using laminarin as assay substrate against the standard curve of glucose. One unit of enzyme activity was defined as µmoles of reducing sugars (D-glucose) released min^{-1} mL^{-1} culture filtrate.

3.2.4.3. β-1, 4-glucanase activity (EC 3.2.1.4; carboxymethyl cellulose (CMCase) activity) (Ghosh et. al., 1983)

Carboxymethyl cellulase (β-1, 4-glucanase) enzyme activity was measured spectrophotometrically by measuring reducing sugars released by hydrolysis of carboxymethyl cellulose (CMC) as substrate (Ghosh et al. 1983). The culture filtrate (0.5 mL) was taken in test tubes. 0.5 ml of 1% caboxymethyl clellulose (CMC) in sodium citrate buffer (0.05M - pH 4.8) was added to the culture filtrate. The tubes were incubated at 50°C for 30 min in waterbath, DNSA solution (3 mL) was added to the tube and incubated at 100°C for 15 min. Reducing sugars liberated were estimated at 575 nm against standard curve of glucose. One unit of enzyme activity was defined as µmoles of reducing sugars (D-glucose) liberated min^{-1} ml^{-1} culture filtrate.

3.2.4.4. Acetylene reduction assay (Hardy et al. 1979)

The acetylene reducing assay (ARA) of cyanobacterial cultures incubated in an atmosphere containing 10% acetylene, was done using gas chromatography of ethylene formed (as an index of nitrogenase activity). Commercially available standard ethylene was utilized for quantification, and vials with an equivalent volume of water served as controls. Glass vials (15 mL capacity) filled with 5 mL of culture suspension were stoppered with air-tight rubber septa (subaseals), incubated under a gas mixture (which had been substituted with 10% acetylene, i.e., 1 mL of air was removed using a syringe with needle, and 1 mL of acetylene was injected in the 10 mL gas space).Such vials were placed under standard growth conditions for 90 minutes. Aliquots of gaseous phase (1 mL) were removed and injected into a preconditioned Bruker Gas Chromatograph model GC 650, housing a two meter long Porapak R stainless steel column and a flame ionization detector. Standard ethylene gas was used for calibration and calculations. All values are expressed as n moles ethylene produced mL culture h^{-1}

3.2.5. Molecular characterization by DNA isolation and fingerprinting

Cyanobacterial cultures (approximately 500 mg wet weight) were collected by centrifugation and total chromosomal DNA was isolated using Ultraclean Plant DNA isolation kit (Mo Bio, USA). Ten primers based on cyanobacteria-specific repeat and/or palindromic sequences (Mazel et al. 1990; Smith et al. 1998) were employed for tracking the strain. Three primer sequences STRR1A 5'-CCA ATC CCC AAT CCC-3', STRRmod 5'-GCG CCC CAA TCC-3'and HIPTG 5'-GCG ATC ACT-3'were selected for PCR amplification based on earlier work (Nayak *et al.*,2009). The total PCR volume was 25 µl with final concentration of constituents: 50 pmol of each primer, 2 mM dNTP, 2.5% (v/w) BSA (Bovine Serum Albumin), 10% (v/v) DMSO (Dimethyl Sulphoxide), 1U of *Taq* DNA polymerase, and*Taq* buffer 2 µl 50-60 ng of DNA.The amplification was carried out with 35 cycles in a Primus 96 Advanced Thermal cycler (peqlab Biotechnologie GmbH, Germany)following the protocol as: pre-denaturation at 95 ^{o}C for 6 min, denaturation at 94^{o}C for 1 min, annealing at 56 ^{o}C for 1 min, extension at 65 ^{o}C for 5 min, and final extension at 65 ^{o}C for 16 min. After amplification, the amplified products were analysed by running in 1.2 % agarose gel prepared using TAE (1×) buffer at 80 V for 2 hours. The PCR amplification was repeated at least three times for each primer to ascertain the reproducibility of the band patterns. The size of bands in all these three primer profiles were measured with the help of AlphaImager software by running 1 Kb DNA ladder obtained from Bangalore Genei Merck (India).

3.3. Experimental set up in lab condition with rice crop under controlled condition with or without cyanobacterial inoculation

3.3.1.Germination of rice seeds

The rice cultivar (cv. *Pusa Sugandh* 5 / Pusa 2511) was obtained from Division of Agronomy, IARI, New Delhi. The rice seeds were surface sterilized with 70% alcohol for 30 sec. and then in 0.1% of mercuric chloride solution for 5min. Later they were rinsed several times with sterile distilled water. Surface-sterilized seeds were kept for germination on the sterilized wet blotting papers.

3.3.2. Co-culturing of rice seedlings with cyanobacterial strain

Rice seedlings (3-4 d old) were transferred into beaker and glass trays containing sterilized 0.8% water agar. The cyanobacterial strain i.e., *Calothrix elenkinii* grown in BG-11-N medium was centrifuged at 10, 000 rpm for 10 min. The chlorophyll concentration was determined and the culture with chlorophyll concentration of 5.0 $\mu gmL^{-1}$was used for inoculation on to water agar.In control, no inoculum and equivalent amount of water was used. The lower portion of trays and beaker was covered with aluminum foil to provide simulated conditions of soil environment. The entire setup comprising two treatments (with or without cyanobacterialculture) was kept in growth chamber, maintained at 27±2 ^{o}C and illumination of 50-55 µmol photons $m^{-2}s^{-2}$ light intensity (16/8h; light/dark cycles). All the treatments were carried out in triplicates.

3.3.3. Plant Parameters

Plant growth parameters, such as root- andshoot lengths, fresh- and dry weights were measured after 7-8 days of transplantation in both the inoculated and un-inoculated seedlings to assess the influence of cyanobacterial cultures on germination and growth of seedlings. Three rice plants were sampled randomly for each treatment.

3.3.4. Plant chlorophyll estimation

The concentration of chlorophyll was estimated spectrophotometrically (Jeffery and Humphrey 1975) after excising the leaves.

3.3.5. Estimation of indole acetic acid (IAA)

The concentration of IAA in roots and shoots was determined spectrophotometrically. Plant samples were washed and ground using sterilized distilled water, followed by methanol addition as an extractant. The samples thus prepared were kept in dark for 30-35 min. and then centrifuged. Plant extract (1 mL) was taken and the Salper Reagent (2 mL, prepared by mixing 1mL of 0.5M $FeCl_3$ in 50 mL of 35% $HClO_4$). Then mixture was incubated in dark for 1h and O.D. was measured at 535nm spectrophotometrically (Gordon and Paleg 1957).

3.3.5. Evaluation of plant defense-and pathogenesis related enzyme activities

Plants were sampled after one week for measuring the activities of defense enzymes such as peroxidase (PO), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL) and pathogenesis related enzyme (CMCase). Three plants were sampled from each replication of the treatment separately and maintained separately for biochemical analysis. Fresh shoot and root samples were washed with tap water followed by deionized water, to remove undesirable materials.The root and shoot samples (1g) were homogenized separately in 1.5 mL of 50 mM Tris HCl Buffer (pH 7.5) at 4°C. The homogenate was centrifuged at 18,000 × g for 20 min. at 4°C. The supernatant was collected in sterilized 2 mLmicrocentrifuge tubes and stored in deep freezer (-20°C); the supernatant was usedas an enzyme source for assaying the activities of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and CMCase. Triplicates samples were taken for each of the assays.

3.3.5.1. Peroxidase (EC 1.11.1.7) activity

Quantitative estimation of peroxidase (PO) activity was done according to method described by Chen et al. (2000). The extract (100 μL) was placed into a spectrophotometer cuvette into which guaiacol (7.5 μL,50 mM in the mixture) and Tris HCl Buffer (792 μL, 0.05 M, pH 6.0) were added. The reaction was initiated by adding 100 μL of 0.6 M hydrogen peroxide and optical density readings were taken at 470 nm. A blank consisting of guaiacol, Tris HCl Buffer and hydrogen peroxide was used to set the spectrophotometer (Spectra max plus 384) at 100% absorbance. One unit of enzyme is defined as change in absorbance (in units) min^{-1} g^{-1} fresh tissue.

3.3.5.2. Polyphenol oxidase (EC 1.14.18.1) activity

Polyphenol oxidase (PPO) was assayed according to Chen et al. (2000) with slight modifications. The reaction mixture contained 0.1 M citrate phosphate buffer, pH 6.0 (1.5 mL), proline (5 mg mL^{-1}), 500 μL; catechol 0.01 M, 500 μL; and 500 μL of enzyme extract. The mixture was aerated before addition of catechol, which served as the substrate. The enzyme activity was determined spectrophotometrically at 546 nm. One unit of enzyme is defined as change in absorbance (in units) min^{-1} g^{-1} fresh tissue.

3.3.5.3.Phenylalanine ammonia lyase (EC 4.3.1.24) activity

Phenylalanine ammonia lyase (PAL) assay was determined spectrophotometrically following the method of Ross and Sederoff (1992). The reaction assay mixture containing 100 µL of enzyme extract and 600 µL of 1 mM L-phenylalanine in 500 µL of 50 mM Tris HCl (pH 8.8), was incubated for 60 min at 40°C. The reaction was arrested by adding 1 mL of 2 N HCl and 1.5 mL of toluene was added, swirled in a vortex for 30 s, centrifuged (400 ×g, 5 min) and the toluene fraction containing trans-cinnamic acid (lower phase) was separated. The absorbance of the toluene phase was measured at 290 nm, against the blank of toluene. The standard curve was drawn with graded amounts of cinnamic acid in toluene as described above. Enzyme activity was expressed as n moles of cinnamic acid min^{-1} g^{-1} fresh tissue.

3.3.5.4.β-1, 4-glucanase activity (EC 3.2.1.4)

Carboxymethyl cellulase (β-1,4-glucanase) enzyme activity was measured spectrophotometrically by measuring the amount of reducing sugars released by hydrolysis of carboxymethyl cellulose (CMC) as substrate (Ghosh et al. 1983). Plant extract (0.5 mL)was taken in test tubes. Carboxymethyl cellulose (CMC, 0.5 mL, 1%) in sodium citrate buffer (0.05M - pH 4.8) was added to it. The tubes were incubated at 50°C for 30 min. in waterbath, 3 mL of DNSA (3,5-Dinitrosalicylic acid) solution was added to the tube and incubated at 100°C for 15 min. Reducing sugars liberated were measured at 575 nm and concentrations were interpolated from the standard curve of glucose. One unit of enzyme activity was defined as µmole of reducing sugars (D-glucose) liberated min^{-1} mL^{-1} culture filtrate.

3.3.6. Electron microscopy of plant samples

Thin sections of roots and shoots (transverse and longitudinal) were prepared. Then sections were dipped in 2.5% Gluteraldehyde (fixative) overnight. After that sections were washed by 0.1M Phosphate buffer for two times. The sections were then processed in different acetone grade i.e, 30%, 50%, 70%, 80%, 90% and 100% and after that they were kept for air drying by adding two drops of Hexamethyl disilazane. Finally sections were coated by Gold palladium in a sputter and observed under electron microscope to study the cyanobacterial association with plants. The Scanning Electron Microscopy facilities in Division of Entomology, IARI, was used for the analyses.

3.3.7. Acetylene reduction assay (ARA) of plant samples (Hardy et al. 1979)

The acetylene reduction assay (ARA) of plant sampleswas done by incubating in an atmosphere, containing 10% acetylene, using gas chromatographic analysis of ethylene formed (as an index of nitrogenase activity). Commercially available standard ethylene was utilized for quantification. Plant samples were kept in 15 mLclean glass tubes and labeled properly. Then 1 mL of sterilized water was added to maintain the humidity in the vials. Water blanks were prepared along with it by taking tube containing 5 mL of sterilized water (as control). Tubescontaining with plant samples were stoppered with air-tight rubber septa (subaseals), and incubated under a gas mixture (which had been substituted with 10% acetylene, *i.e.*, 1 mL of air was removed using a syringe with needle, and 1 mL of acetylene was injected in the 10 mL gas space).Such tubes were placed under growth chamber for 3 h. Aliquots of gaseous phase (1 mL) were removed and injected into a preconditioned Bruker Gas Chromatograph model GC 650, housing a two meter long Porapak R stainless steel column and a flame ionization detector. Standard ethylene gas was used for calibration and calculations. All values are expressed as n moles ethylene produced mL culture mg chl. Each measurement was done in triplicate.

3.4. Enumeration and isolation ofculturable endophytic bacteria

3.4.1. Population density of culturable endophytes

Root- and shootsamples were plated on eight different media using standard spread plate technique in order to enumerate nitrogen fixers and phosphate solublizers by incubating suitable temperature for appropriate time.Different dilutions of root and shoot extracts were prepared in normal saline (0.8% NaCl). Eight different media used were: (i) Yeast Extract Mannitol Agar,(ii) Pikovskaya's Agar,(iii) Soil Extract Agar, (iv) R2A (Reasoner's 2A agar) medium, (v) Jensen's Agar, (vi & vii) BG-11 (+N and –N), (viii) N-free Semi Solid Agar, and (ix) N-free Malate medium (Details of media used is given in Appendices). After desired periods of incubation, the growth of microbial colonies on different media plates was observed and the numbers of microbial colonies were enumerated. The colony morphology and total viable counts were recorded for the both root and shoot samples on different media employed. Based on the differences in colony morphologies, the representativemorphotypes were selected for further analysis.

3.5. *Molecular characterization of the unculturable microbial diversity*

3.5.1. *Isolation of genomic DNA*

Plant root- and shootsamples of each treatment were extracted for DNA by using Ultraclean Plant DNA isolation kit (MoBio Inc, USA). Rice plants were rinsed in sterile distilled water to remove soil particles and then the root and shoot portions were separated. The shoot and root plant samples (0.5 g) were crushed in sterilized mortar-pestle using sterile water and the processed tissues were used for extracting the DNA as per the manufacturer's instructions.

3.5.2. *PCR amplificationof 16S rRNA gene*

Amplification of 16SrRNAwas done using primers specific for cyanobacteria [fD2 (5'-AGAGTTTGA TCATGGCTCAG-3') and rD19 (5'-AAGGAGGTGATCCAGCCGCA-3')]to isolate the nearly complete 16S rRNA gene sequence (Weisburg et al., 1991).The reaction mixture contained 1× Taq buffer, 2.5 mM $MgCl_{2,}$ 0.3mM, each of the deoxynucleotide triphosphate, 10 pmol of each primer, 1U *Taq* DNA polymerase, 50 ng of template DNA and MilliQ to give final volume of 25 µL. The reaction mixture were incubated for amplification in a Primus 96 Advanced Thermal cycler (peqlab Biotechnologie GmbH, Germany) using the optimized program as described by Hisbergues et al. (2003). The PCR conditions consisted of initial denaturation of 92°C for 5 min., 35 cycles of 92°C (30 sec.), 64°C (45 sec.), and 72°C (2 min.), plus one additional cycle with afinal 10 min. chain elongation. All PCR related chemicals were purchased from Bangalore Genei Merck Pvt. Ltd., India. PCR amplification and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al., 1989). DNA ladders were purchased from MBI Fermentas and all PCR related chemicals from Bangalore Genei Pvt. Ltd., India. The PCR products were analyzed in horizontal 1.2% (w/v) agarose gel (0.5 mg ethidium bromide mL^{-1}) in TAE buffer 20 mM Tris-acetate, 0.5 mM EDTA (pH 8.0). The molecular weight standard was a 1kbp ladder (Fermentas). The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California).

3.5.3. PCR Amplification of 16SrRNAusing degenerate bacterial primers for DGGE analysis

The 16S rRNA PCR fragments were amplified using specific primers 341F with GC-clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTC CCG CCG CCC CCGCCC GCC TACGGGAGGCAGCAG-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer et al.1993). The reaction mixture contained 1× Taq buffer, 2.5 mM $MgCl_{2,}$ 0.3mM, each of the deoxynucleotide triphosphate, 10 pmol of each primer, 1U *Taq* DNA polymerase, 50 ng of template DNA and MilliQ to give final volume of 25 µL. The reaction mixture were incubated for amplification in a PEQlab Primus 96 Thermal cycler using the optimized program as described by (Hisbergues et al., 2003). The PCR conditions consisted of initial denaturation of 94°C for 2min., 35 cycles of 94°C (30 sec.), 59°C (1 min.), and 72°C (2 min.), plus one additional cycle with a final 8 min. chain elongation at 72°C.The PCR amplification and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al., 1989). The PCR products were analyzed in horizontal 1.2% (w/v) agarose gel (0.5 mg ethidium bromide mL^{-1}) in TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0). The molecular weight standard was a 1kbp ladder (Fermentas). The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California). The PCR products were stored at20°Cbefore DGGE analysis.

3.5.4. *Analysis of PCR products by denaturing gradient gelelectrophoresis (DGGE)*

All reagents were prepared as described in the Bio-Rad D Gene Instruction Manual and Applications Guide (Bio-Rad, Hercules, CA). The DGGE gels were run using a Bio-Rad DCodeTM Universal Mutation Detection System (Bio-Rad, Hercules, CA). Reagents used were 40% Acrylamide/Bis(37.5:1)(acrylamide, 38.93g, Bis-acrylamide, 1.07g mixed with 100mL of deionized water), which wasfiltered through 0.45µm filter and stored at 4^{o}C. For the 50× TAE Buffer (pH 8.0), Tris base (242g), glacial acetic acid (57.1 mL), and EDTA (0.5M) were added to 1000mL of deionized water. Then the mixture was autoclaved for 20-30 min. and stored at room temperature before used.Thegels used for DGGE were 6% polyacrylamide gel (40%-acrylamide and N,N-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1× TAE) containing a linear gradient of the denaturant concentration ranging from 30% to

70% with 1 mm thick. The denaturing gradient gel was run for 6 h at 60°C and 150 V. After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 mg mL^{-1}). The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California).

The respective primers were used to carry out the forward and reverse DNA sequencing using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence was generated from forward and reverse sequence data using the aligner software (BioEdit). The 16S region sequence was used to carry out BLAST with the nr database of NCBI Genbank database. Based on the maximum identity scores, the representative sequences were selected for the construction of the phylogenetic tree using MEGA 6.

3.6. Effect of cyanobacterial inoculation on microbiota of rice

3.6.1. Experimental Conditions

The rice cultivar (cv. Pusa Sugandh-5 (Pusa 2511)) was collected from the Division of Genetics, IARI, New Delhi. The soil samples for the pot experiments were collected the experimental fields of IARI, air-dried and processed before use. The soil was of sandy loam type with pH, and electrical conductivity (EC, dS m^{-1}). Pot experiments were carried out under thecontrolled environmental conditions of National Phytotron Facility, IARI, New Delhi. Pots of 10” size were filled with soil samples (8 kg). For the experiment with soil under sterile conditions, a set of filled-in pots were autoclaved at 1.05 kg cm^{-2} pressure and 121°C temperature for one hour on three consecutive days. Another set of filled-in pots with unsterile soils was also maintained in the environmental chamber.

3.6.2. Co-culturing of rice seedlings with cyanobacterial strain in pots

Rice seedlings of 2-week old were used for transplantation at five seedlings per pot. For the inoculation with*Calothrix elenkinii*, the cyanobacterial culture with the chlorophyll concentration of 0.89µg g^{-1} was added to both the sets of sterile and unsterile pots. The treatments used under the study were:

Sterile soil: T1- Rice Plant (control)

T2- Rice Plant + *Calothrix elenkinii*

Unsterile soil: T1- Rice Plant (control)

T2- Rice Plant + *Calothrix elenkinii*

A total of 10 pots were maintained for each treatment. The rice plants were grown under controlled environmental conditions (30°C/25°C with light:dark cycles (i.e., day/night of 14/10 h), relative humidity maintained at 70% throughout the growth period).

3.6.2.1. Soil and plant sampling for analyses

Both soil (bulk- and rhizospheric) and plants were sampled at 15 and 45 DAT (Days after Transplanting) for soil-, plant biometric-, and molecular microbial analyses. All the samples were collected in triplicates.

I. Soil parameters

A. Nutrient analysis of soil: Different available nutrients in soil was estimated by using these methods-

I. Estimation of Available N in soil by alkaline permanganate ($KMnO_4$)
(Subbiah and Asija, 1956)

II. Estimation of available phosphorus (P) in soil (Pkg ha^{-1})
(Olsen et al., 1952)

III. Estimation of available potassium (K) in soil (K kg ha^{-1})
(Flame photometer method, Jackson, 1973)

V. Estimation of organic carbon (%) in soil
(Walkley and Black, 1934)

VI. Soil pH (1:2.5, soil:water)
(Elico pH meter, Piper, 1950)

B. Soil chlorophyll (Nayak et al. 2004)

Soil chlorophyll was assayed using fresh soil cores, collected with the help of tube augar (from 0-20 cm depth), which was placed in 55 ml glass test tubes. Acetone: DMSO (1:1) mixture (10 mL) was added to soil at a rate of 4 ml g^{-1} soil and tightly sealed using subaseal stoppers. The contents were thoroughly shaken and the tubes were incubated in dark at room temperature for 48-96 h, until all the pigments get extracted. Intermittent shaking is done after every 24 h to extract chlorophyll completely. If 10 ml acetone: DMSO mixture is consumed after 24 h then more 10 ml mixture is added to the soil of the tubes. The colored solvent is removed, centrifuged to remove debris and optical density values were taken at 663, 645 and 630 nm, using solvent mixture as control. The calculations were done using formula, given below:

Soil chlorophyll (μg g^{-1}) = 11.64 (OD_{663}) – 2.16 (OD_{645}) + 0.10 (OD_{630})

C. Acetylene reduction assay (Prasanna et al. 2003)

Acetylene reducing activity (ARA) in soil cores incubated in an atmosphere, containing 10% acetylene, was estimated using gas chromatography of ethylene formed (as an index of nitrogenase activity) and expressed as acetylene reducing activity (ARA). Commercially available standard ethylene was utilized for quantification, and vials with an equivalent volume of water served as controls. 50 mL glass tubes filled with soil of one augar, were incubated under a gas mixture which had been substituted with 10% acetylene under standard growth conditions for 1 and 1/2 h. One ml aliquots of gaseous phase were removed and injected into a preconditioned gas chromatograph, housing a two meter long Porapak R stainless steel column and a flame ionization detector. The column temperature was maintained at 100°C and injector and detector at 110°C. A flow rate of 35 ml min^{-1} of N_2 served as the carrier gas. Standard ethylene gas was used for calibration and calculations. All values presented are the means of triplicate measurements and expressed as n moles ethylene produced g^{-1} soil h^{-1}.

D. Analyses of cellular fatty acids of culturable microbial isolates (FAME)

Fatty acids from the culturable microbial isolates were extracted using the method of Sasser (1990). Briefly, the bacterial cells were gently scraped from the surface of agar, transferred to a Teflon-lined screw-cap tube, and saponified by heating them at 100 °C for 30 min following the addition of NaOH (1 mL, 15% in 50% aqueous methanol). After cooling the hydrolysate, methanolic HCL (2 mL) was added and the mixture was heated to 80 °C for 10 min. The methylated fatty acids were cooled immediately and extracted through the addition of hexane-methyl-*tert*-butyl ether (1.25 mL, 1:1 vol/vol) with mixing. After removing the lower aqueous layer carefully, dilute NaOH was added to the remaining organic layer for clarification of the phase interface. The organic layer containing the fatty acid methyl esters (FAMEs) were analysed using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, U.S.A.). The gas chromatograph was fitted with a 5% phenyl-methyl silicone capillary column and a flame ionization detector. The peaks were automatically integrated and fatty acid names and percentages calculated by the software (MIDI Inc Sherlock MIS, version 6.1).

The identification of microorganisms was performed by comparing the unknown organism's FAME profile with the profiles in the library. The library matches are ranked by similarity and the library entry which is closest match to the unknown organism's FAME profile is ranked the highest, followed by the next analogous entry. The profiles of the library organisms were established on the basis of a multivariate Gaussian model of at least 12 strains of each species. The correlation of an unknown organism's profile with a library entry was expressed as a similarity index (SI) on a numeric scale of 0 to 1.0. While the SI values of $\geq$ 0.6 were considered excellent matches, a value of 0.5 represented approximately three standard deviations from the library profile mean. The microorganisms with SI values of $\leq$0.50, identified using the aerobic library (RTBSA6 6.21), were also included in the study.

E. Enrichment culture analysis:

Bulk soil samples from the treatments were inoculated in different bacterial culture medium (Nutrient broth, Jensen's broth, Pikovskya's, broth, Yeast tryptone broth, Yeast mannitol broth, and BG-11(+N and –N) for enrichment of different bacteria. Further DNA isolation of enriched culture was done to study their diversity.

II. Microbial community analyses

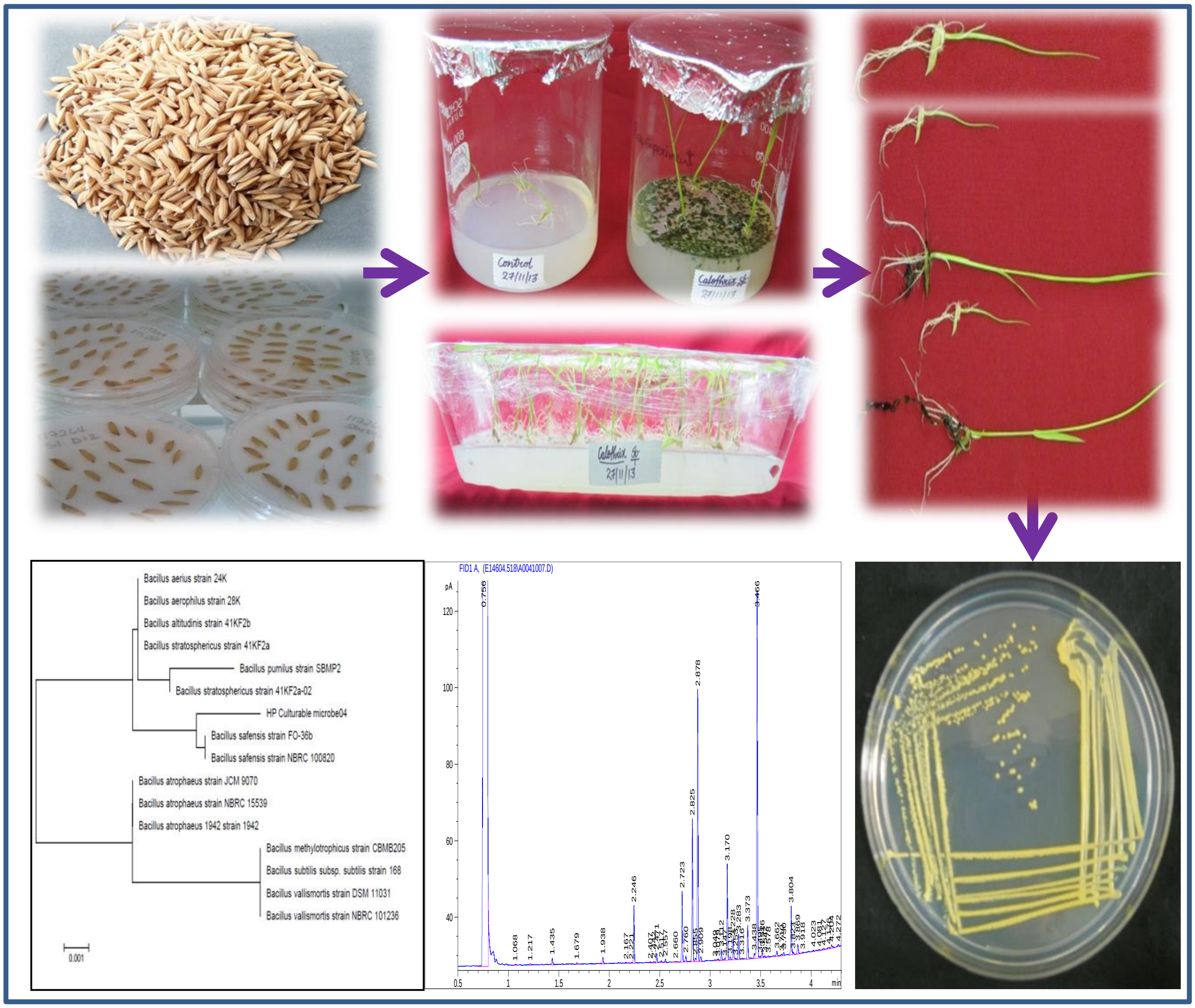
Isolation of genomic DNA for DGGE profiling

Plant root- and shoot samples of each treatment were extracted for DNA by using Ultraclean Plant DNA isolation kit (MoBio Inc, USA). Rice plants were rinsed in sterile distilled water to remove soil particles and then the root and shoot portions were separated. The shoot and root plant samples (0.5 g) were crushed in sterilized mortar-pestle using sterile water and the processed tissues were used for extracting the DNA as per the manufacturer's instructions. Samples from bulk soil were set up as enrichment cultures in different media- Yeast Extract Mannitol Broth and Pikovaskaya Broth at different (10^1 and 10^6) and used for DNA extraction after one week using ZR Fungal / Bacterial DNA Miniprep Kit (Zymo Research Corp., USA). The mixtures of selected cyanobacterial strains available from the germplasm of the Division of Microbiology, IARI, New Delhi were also used as additional reference samples for DGGE profiling, as given earlier.

Analysis of bulk- and rhizosphere soil PLFAs

The standard protocols of Zelles and Bai (1993) and Buyer et al. (2010) were followed for the PLFA analysis. Briefly, the lipid fractions were extracted from 5 g of fresh soil using a modified Blight-Dyer extraction with 19 mL of extractant. After evaporation under a stream of nitrogen, lipids were separated on a solid-phase extraction column. Later, these fractions were eluted with 5 mL of methanol. After evaporation under nitrogen, the phospholipids were transesterified to fatty acid methyl esters, extracted with hexane, evaporated and analyzed by gas chromatography GC, equipped with an autosampler, and flame ionization detector. Fatty acid methyl esters (FAMEs) were separated on a 25 m long × 0.2 mm internal diameter × 0.33 µm film thickness. Initial oven temperature was 190 ^{o}C, ramping to 285 ^{o}C at 10 ^{o}C/min and then to 310 ^{o}C at 60 ^{o}C/min, followed by a hold at 310 ^{o}C for 2 min. Injector temperature was 250 ^{o}C and detector temperature was 300 ^{o}C. The MIS Sherlock (MIDI, Inc, Newark, DE, USA) was used to control the system and FAMEs were identified using the MIDI PLFAD1 calibration mix and naming table. An internal standard of methyl nonadecanoate allowed the calculation of FAME concentrations.

Different fatty acid types which were obtained by the conversion of raw fatty acid data by the Sherlock software included straight, branched, hydroxyl, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), dimethyl acetal and other mixed functional groups. Total PLFAs of different fatty acids were calculated using both absolute (nmole g^{-1} soil) and relative abundances (mole percent of $PLFA_{Total}$). In addition, these fatty acids were also summed into different ‘Biomarker groups’ as suggested by Frostegard and Baath (1996), Zelles (1999) and Ringelberg et al. (1997). The summed masses of PLFAs typical of Gram-positive-, and Gram-negative bacteria, actinobacteria, anaerobe, fungi and eukaryotes were also calculated for quantifying both absolute and relative abundances.



4. RESEARCH PAPER I

4. RESEARCH PAPER I

Influence of cyanobacterial inoculation on the culturable diversity of rice microbiome

Abstract

Cyanobacteria are ubiquitous photosynthetic bacteria and are primary colonizers of various environmental niches. They can colonize roots and elicit growth promotory- and defense responses in rice. The present investigation was aimed at examining the responses in terms of plant growth and enzyme activities, changes in the microbiome, with emphasis on culturable members, and the presence of inoculated cyanobacterium (*Calothrix elenkinii*) in roots of rice (cv. *Pusa Sugandh 5*) grown under the controlled condition. The biometric- and biochemical analyses of root and shoot tissues revealed significant increases in plant growth parameters (15 vs. 25 mg dry weight), nitrogenase activity (2.94 vs. 11.11 µ moles of ethylene plant^{-1} h^{-1}), and indole acetic acid (IAA) production (1.87 µg mL^{-1} vs. 2.66 µg mg^{-1} root tissue; control vs. treatment). Hydrolytic enzyme activities such as β-1,4-endoglucanase and chitosanase increased in the cyanobacterial inoculated plants. Likewise, the activities of defense enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase enhanced. The enumeration of culturable microorganisms from the roots and shoots suggested comparable amounts in both the control and treated plants, probably due to the bias in culturing. The cultural microorganisms isolated using nitrogen-free media (Yeast Extract Mannitol and Jensen's media), and Pikovskaya's medium showed their potential for nitrogen fixation and phosphorus solubilisation, respectively. Fatty acid methyl ester (FAME) analysis of the isolates selected to represent different morphological diversity suggested the predominance of the members of Bacillaceae. Similar observation was made by the16S rRNA sequencing of selected morphotypes. Although different media with varying concentrations of nutrients were used to culture microorganisms from rice, the members of *Bacilliaceae* were predominant under the conditions tested. The scanning electron microscopic observations showed visible enhancement in the number of rod-shaped bacterial cells in the *Calothrix* sp. inoculated roots, relative to the control. Molecular identification

using the primers of STRRmod and HIP-TG confirmed the presence of *Calothrix elenkinii*, only in the inoculated plants.

Introduction

The nature of plant-microbe interactions is based on the type of microorganisms involved, which determines the final outcome of the relationship, ranging from pathogenesis to symbiosis, or even benign association. Most of these interactions alter the nutrient dynamics and susceptibility to pathogens, besides influencing growth and development of plants (Dobbelare *et al.* 2001). In an evaluation of microbiota under controlled conditions, the metabolic activity and cell wall features of plant cells are found to play a critical role in the host plant's selection of soil bacteria for colonization (Bulgarelli *et al.* 2012). The fungal and bacterial populations in soil are able to detect and decipher the plant signals and colonize the rhizosphere by producing plant growth regulating substances such as auxins and cytokinins; these microbial colonisers can elicit immune responses in plants. Alternatively, plants also recognize the microbe-derived compounds and modulate their defense and growth responses, based on the type of microorganisms encountered (Glick 1995). Thus, the rhizosphere supports and mediates highly coordinated and intricate mechanisms and cellular processes of both plants and microorganisms (Bais *et al.* 2004). The rhizosphere of most plants is often considered to be one of the richest microbial ecosystems on Earth (Hirsch and Mauchline 2012).

Cyanobacteria represent a promising group of photosynthetic bacteria which are ubiquitous and primary colonizers in various environments. Their close evolutionary relationships with plants combined with eubacteria related properties have made them the subject of research worldwide. Cyanobacteria are well adapted to a diverse range of environmental conditions and are employed as inoculants for enhancing soil fertility and improving soil structure besides enhancing crop yields, especially in rice (Venkataraman 1972; Kaushik 1998; Nayak *et al*. 2004; Dhar *et al.* 2007). They also add organic matter, synthesize and liberate amino acids, vitamins, auxins, reduce oxidizable matter content of the soil, provide oxygen to the submerged rhizosphere, ameliorate salinity, buffer the pH, solubilize phosphates and facilitate nutrient use efficiency in crop plants (Mandal et al. 1998; Kaushik 1998). Scattered reports are available on the colonization of cyanobacteria on rice and wheat roots and elicitation of defense responses, which indicate an intimate interaction of cyanobacteria with the plants (Babu et al. 2014; Gantar et al. 1991; Svircev et al. 1997;

Karthikeyan et al. 2009; Prasanna et al. 2008, 2009a,b; 2011, 2013a,b,c). However, their relationship on the rice microbiome is less explored. The present investigation is aimed at analyzing the plant microbiome (microbial communities in roots and shoots) in the presence and absence of cyanobacterial inoculation in rice seedlings under controlled conditions. Employing cultural approaches complemented by microscopic studies, biochemical and molecular tools, the investigation aims at gaining a better understanding of the effect of cyanobacterial inoculation on functional attributes of rice plants and identify major microbial players of the rice microbiome.

Materials and Methods

Growth and maintenance of *Calothrix elenkinii*

The axenic culture of *Calothrix elenkinii* (RPC1) was obtained from the germplasm Collection of the Division of Microbiology, IARI, New Delhi. The purity of culture was examined by microscopic observation at regular intervals. The strain was grown and maintained using BG-11(-N) medium (Stanier et al., 1971), at 27±2°C under light: dark cycles (16:8 h) and intensity of 50-55 µmol photons $m^{-2}s^{-2}$ using cool white fluorescent tubes. This strain has been characterized earlier in several laboratory and field studies (Karthikeyan et al. 2009; Prasanna et al. 2013a,b, 2014a) for its plant growth promoting properties and its ability to produce hydrolytic enzymes and exhibit biocontrol against phytopathogenic fungi (Manjunath et al. 2010; Natarajan et al. 2012, 2013; Prasanna et al. 2014a,c; Radhakrishnan et al. 2009).

Evaluation of growth and physiological attributes of *Calothrix elenkinii*

The culture was inoculated at a rate of 10% (v/v) in 250 mL conical flasks, containing 125 mL sterile BG11 medium. The activity of enzymes- chitosanase, endoglucanase (β-1, 3 - glucanase) and carboxy methyl cellulase (CMCase / β-1, 4 - endoglucanase) activity were assayed spectrophotometrically using glycol chitosan, laminarin and carboxymethyl cellulose respectively, as the substrates. One unit (IU) of chitosanase, endoglucanase and CMCase activity represents one µmole of end product released mL^{-1} min^{-1}., under the assay conditions (Prasanna et al. 2008; 2013b). The concentration of IAA in the culture filtrates was determined spectrophotometrically by the method outlined by Gordon and Pelag (1957) using IAA as standard.

Nitrogenase activity was assayed using 5 mL samples of homogenized suspensions of 2 weeks old cultures taken in glass vials. In each tube, ten percent air space (v/v) was replaced with acetylene as the substrate for nitrogenase enzyme. The tubes were incubated for 24 h under a temperature of 27± 1 °C , L : D (light : dark cycles 16 : 8), white light (50 – 55 µmol photons m^{-2} s^{-1}) as static cultures. Appropriate controls, without acetylene gas injection, the controls were maintained. Three replications per treatment were maintained. After incubation, 0.1 mL of the gas sample was removed from the tubes and injected into a Gas chromatograph (Model Bruker 450 with FID detector) for analysis. Standard ethylene gas was used for calibration and calculations. The ethylene produced by the reduction of acetylene was calculated and expressed as nmoles ethylene produced mg $chlorophyll^{-1}$ h^{-1}. For the bacterial isolates, the 48 h grown slants of N-free medium were incubated for 24h in glass vials injected with 10% acetylene and the values expressed as nmoles of ethylene produced per vial h^{-1}.

Experimental set up with rice crop under controlled conditions

Germination of rice seeds

The rice cultivar cv. *Pusa Sugandh 5* (Pusa 2511) was obtained from Division of Agronomy, IARI, New Delhi. The rice seeds were surface sterilized with 70% alcohol for 30 s and then in 0.1% of mercuric chloride solution for 5 min. Then they were rinsed several times with sterile distilled water. Surface-sterilized seeds were kept for germination on the sterilized wet blotting sheets.

Co-culturing of rice seedlings with cyanobacterial strain

Rice seedlings (3-4 d old) were transferred into beakers and glass trays containing sterilized 0.8% water agar (Fig. 1). The cyanobacterial strain, i.e., *Calothrix elenkinii* grown in BG-11-N medium was centrifuged at 10,000 rpm for 10 min. The chlorophyll concentration was determined, and the culture with chlorophyll concentration of 5.0 µg mL^{-1} was used for inoculation onto water agar. In the control, no inoculum but with the equivalent amount of water was used. The lower portion of trays and beaker was covered with aluminum foil to provide simulated conditions of the soil environment. The entire setup comprising two treatments (with or without the cyanobacterial culture) was kept in the growth chamber,

Fig. 1 Experimental setup with rice seedlings grown in water agar under controlled conditions. Control and inoculated (*Calothrix* sp.) treatments

maintained at 27±2^{o}C and illumination of 50-55 μmol photons $m^{-2}s^{-2}$ light intensity (16/8 h; light/dark cycles). All the treatments were carried out in triplicates.

Plant Parameters

Plant growth parameters, such as root- and shoot length, fresh and dry weight were measured using 10 d old seedlings, in both the inoculated and un-inoculated seedlings to assess the influence of cyanobacterial cultures on germination and growth of seedlings. Three rice plants were sampled randomly for each treatment.

Plant chlorophyll estimation

The chlorophyll content of root and shoot tissues were determined after extraction of chlorophyll in DMSO (Dimethyl sulphoxide) using a standard methodology, as optimized earlier (Jeffrey and Humphrey 1975), spectrophotometrically using a hot extraction method. Triplicate samples of the media (10 mL) were centrifuged at 3000 rpm for 10 min. The pellet was transferred to a test tube, and 10 mL of 95% methanol was added. The test tubes were kept in the water bath at 60°C for 30 min. The mouth of the tubes was covered to prevent evaporation of the solvent. The tubes were removed from the water bath, cooled at room temperature and made up the volume of the solvent if any lost. The tubes were centrifuged at 3000 rpm for 10 min, and OD was taken for the supernatant at 650 and 665 nm. The chlorophyll content was expressed as μg mL^{-1} (Sood *et al.* 2011).

Estimation of indole acetic acid (IAA) and ARA (as an index of nitrogen fixing potential)

The concentration of IAA in roots and shoots was determined spectrophotometrically (Gordon and Paleg 1957). Plant samples were washed, and ground using sterilized distilled water, followed by methanol addition as an extractant. The samples were kept in dark for 30 min and centrifuged. Plant extract (1 mL) was taken and the Salper Reagent (2 mL, prepared by mixing 1mL of 0.5M $FeCl_3$ in 50 mL of 35% $HClO_4$). Then mixture was incubated in dark for 1h, and O.D. was measured at 535 nm spectrophotometrically.

Acetylene reduction activity (ARA), as an index of nitrogenase activity of the whole plants, was measured using the methodology described in Prasanna *et al*. (2013 b). A single plant was placed in a glass vial (15 mL) and sealed with air-tight rubber stoppers and analyses undertaken as given using Gas Chromatograph. The values were expressed as nmoles of ethylene produced per plant h^{-1}.

Evaluation of plant defense-and pathogenesis-related enzyme activities

The fresh roots were washed in running tap water and homogenized with 5 mL of 50 mM Tris-HCl buffer using a mortar and pestle. The tissue extracts were centrifuged at 14,000 g for 20 min at 4 °C, and the supernatant was transferred to 5 mL vials and stored at -20 °C. Polyphenol oxidase (PPO) activity was measured using the catechol as the substrate. Aliquots of 500 µL were taken in 2 mL cuvette containing 1mL of 0.02 M citrate phosphate buffer, pH 6.0 ; proline (5 mg mL^{-1}), 0.5 mL; catechol (2 mg mL^{-1}) 0.5 mL; and 500 µL of enzyme extract. The mixture was aerated before addition of catechol. The enzyme activity was determined spectrophotometrically at 546 nm. The changes in absorbance at 546 nm were recorded at 30 s intervals for 3 min. One unit of enzyme was expressed IU g^{-1} fresh weight. Phenylalanine ammonia lyase (PAL) activity was assayed in leaf and root extracts (100 µL) using 2.5 mL of 0.2 % L-phenylalanine in 50 mM Tris-HCl (pH 6) buffer solution. The reaction mixture was incubated at 40 °C for 60 min. The amount of trans-cinnamic acid formed from L-phenylalanine was measured spectrophotometrically at a wavelength of 290 nm against the blank. In the blank, 0.1 mL of distilled water is used in place of test sample. Enzyme activity was expressed as nmoles of cinnamic acid h^{-1} g^{-1} fresh weight. Peroxidase (PO) activity was also measured using guaiacol (molar extinction coefficient 26.6 mM^{-1} cm^{-1}) as the hydrogen donor. Aliquots of 1 mL were taken in 1.5 mL cuvette containing 1% (v/v) guaiacol in 0.01 M sodium phosphate buffer (pH 6.0) and 0.1 M H_2O_2. The enzyme extract was added to initiate the reaction. The changes in absorbance at 470 nm were recorded at 30 s intervals for 3 min. One unit of enzyme is expressed IU g^{-1} fresh weight. All the procedures were modified and optimized as given in Prasanna et al. (2013b).

The activity of enzymes- chitosanase, endoglucanase (β-1, 3 - glucanase) and carboxy methyl cellulase (CMCase / β-1, 4 - endoglucanase) were assayed spectrophotometrically using glycol chitosan, laminarin and carboxymethyl cellulose respectively, as the substrate. One unit of chitosanase, endoglucanase and CMCase activity represents IU g^{-1} fresh weight, under the assay conditions (Prasanna *et al.* 2008; 2013b).

Electron microscopy of plant samples

Scanning electron microscopy (SEM) of root and shoot sections was undertaken, using sterilized root/shoot bits (after washing with 0.1 % mercury (II) chloride for 1 min and thereafter with 70% ethanol for 30 s, followed by thorough washing using sterile water). Triplicate samples were observed. Such samples were cut, prefixed overnight in 2.5% (v/v) glutaraldehyde, washed using 0.1 M sodium phosphate buffer of pH 6.9 for 10-15 min (3 times), and 1% (w/v) osmium tetraoxide (OsO_4) was used for post fixation. The fixed cells were dehydrated by sequential passage through increasing concentrations of acetone 30-100% (v/v) with 20% increments upto dry acetone. The samples were dried in a critical point dryer (saturated with CO_2 at a temperature of 40 ^{0}C under the pressure of 70 atm), coated with gold palladium for 60 sec in a Pelco 3 sputter coater, and visualized under Scanning Electron Microscope (Evo Maio Zeiss, Germany).

Enumeration and analyses of culturable bacterial diversity from root and shoot tissues

Population density of culturable bacteria

Dilutions of root and shoot samples were prepared in normal saline (0.8% NaCl) and suitable aliquots plated on eight different media using standard spread plate technique in order to enumerate the bacteria present and also evaluate the nitrogen fixers and phosphate solublizers (Figure showing type of colonies given in Appendices). The different media used were: (i) Yeast Extract Mannitol Agar, (ii) Pikovskaya's Agar, (iii) Soil Extract Agar, (iv) R2A (Reasoner's 2A agar) medium, (v) Jensen's Agar, (vi & vii) BG-11 (+N and –N), (viii) N-free Semi Solid Agar, and (ix) N-free Malate medium. After period of incubation, the number of colonies was enumerated. The colony morphology and total viable counts were recorded for the both root and shoot samples on different media employed. Based on the differences in colony morphologies, representative morphotypes were selected for further analysis (Figure showing morphotypes selected for FAME analyses given in Appendices).

Analysis of cellular fatty acids of culturable microbial isolates

Fatty acids from the culturable microbial isolates were extracted using the method of Sasser (1990). Briefly, the bacterial cells were gently scraped from the surface of agar, transferred to a Teflon-lined screw-cap tube, and saponified by heating them at 100 ^{o}C for 30 min following the addition of NaOH (1 mL, 15% in 50% aqueous methanol). After cooling the hydrolysate, methanolic HCL (2 mL) was added and the mixture was heated to 80 ^{o}C for 10 min. The methylated fatty acids were cooled immediately and extracted through the addition of hexane-methyl-*tert*-butyl ether (1.25 mL, 1:1 vol/vol) with mixing. After removing the lower aqueous layer carefully, dilute NaOH was added to the remaining organic layer for clarification of the phase interface. The organic layer containing the fatty acid methyl esters (FAMEs) were analysed using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, U.S.A.). The gas chromatograph was fitted with a 5% phenyl-methyl silicone capillary column and a flame ionization detector. The peaks were automatically integrated and fatty acid names and percentages calculated by the software (MIDI Inc Sherlock MIS, version 6.1).

The identification of microorganisms was performed by comparing the unknown organism's FAME profile with the profiles in the library. The library matches are ranked by similarity and the library entry which is the closest match to the unknown organism's FAME profile is ranked the highest, followed by the next analogous entry. The profiles of the library organisms were established on the basis of a multivariate Gaussian model of at least 12 strains of each species. The correlation of an unknown organism's profile with a library entry was expressed as a similarity index (SI) on a numeric scale of 0 to 1.0. While the SI values of $\geq$ 0.6 were considered excellent matches, a value of 0.5 represented approximately three standard deviations from the library profile mean. The microorganisms with SI values of $\leq$0.50, identified using the aerobic library (RTBSA6 6.21), were also included in the study.

Isolation of genomic DNA

DNA extraction from plant root and shoot samples of each treatment was undertaken using Ultraclean Plant DNA isolation kit (MoBio Inc, USA). Rice plants were rinsed in sterile water to remove soil particles and then the root and shoot portions were separated. The shoot and root plant samples (0.5 g) were crushed using the sterilized mortar-pestle using sterile water, and the processed tissues were used for extracting the DNA as per the manufacturer's instructions.

PCR amplification of 16S rDNA

Amplification was done using primers specific for cyanobacteria [fD2 (5'-AGAGTTTGA TCATGGCTCAG-3') and rD19 (5'-AAGGAGGTGATCCAGCCGCA-3')] to isolate the nearly complete 16S rDNA gene sequence (Weisburg et al. 1991). For all the bacterial isolates, primers used for amplification of 16S rRNA gene from the genomic DNA of isolates were pA (5′AGA GTT TGA TCC TGG CTC AG3′) and pH (5′-AAG GAG GTG ATC CAG

CCG CA-3′) (Edwards et al. 1989). The reaction mixture contained 1× Taq buffer, 2.5 mM $MgCl_{2,}$ 0.3mM, each of the deoxynucleotide triphosphate, 10 pmol of each primer, 1U *Taq* DNA polymerase, 50 ng of template DNA and MilliQ to give final volume of 25 µL. The reaction mixture were incubated for amplification in a Primus 96 Advanced Thermal cycler (Peqlab Biotechnologie GmbH, Germany) using the optimized program as described by Hisbergues et al. (2003). The PCR conditions for cyanobacterial 16 rDNA amplification consisted of initial denaturation of 92°C for 5 min., 35 cycles of 92°C (30 sec.), 64°C (45 sec.), and 72°C (2 min.), plus one additional cycle with a final 10 min. chain elongation. Conditions used for amplification of bacterial 16S rDNA were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. All PCR related chemicals were purchased from Bangalore Genei Merck Pvt. Ltd., India. PCR amplification and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al. 1989). DNA ladders were purchased from MBI Fermentas and all PCR related chemicals from Bangalore Genei Pvt. Ltd., India. The PCR products were analyzed in horizontal 1.2% (w/v) agarose gel (0.5 mg ethidium bromide mL^{-1}) in TAE buffer 20 mM Tris-acetate, 0.5 mM EDTA (pH 8.0). The molecular weight standard was a 1kbp ladder (Fermentas). The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California).

Genotypic analysis

The cyanobacterial filaments (approximately 50 mg wet weight) were pelleted and washed four times with sterile Milli Q water. The samples were vortexed and repeatedly crushed using mini pestle, in order to rupture the filaments. The cell pellet was resuspended in 5 µL of MilliQ water and used directly as a template for PCR (Nayak *et al.* 2009). The primer HIP-TG (5’ - 3’ GCGATCACTG), was used for PCR amplification. The primers were synthesized by Bangalore Genei (India). The PCR volume was 25 µL, containing 50 pmol of each primer, 2 mM dNTP, 2.5% (v/w) BSA, 10% (v/v) DMSO, 1U of *Taq* DNA polymerase, *Taq* buffer and 5 µL of cyanobacterial filaments. The amplifications were performed in a Peqlab QB96 thermocycler as follows: one cycle at 95°C for 6 min, 35 cycles at 94°C for 1 min, 56°C for 1 min, 65°C for 5 min, and 65°C for 16 min, with the final step at 4°C. The

PCR amplification products were resolved on 1.5% agarose gel prepared using TAE (Tris acetate EDTA; 1X) buffer and electrophoresis was done at 80 V for 2 h. The PCR reactions were repeated at least three times to ascertain the reproducibility of the band profiles. The size of bands in the profiles were determined by comparison with the profiles of standard molecular markers (1 kb ladder), procured from Bangalore Genei (India).

The 16S rRNA sequences of selected culturable bacteria were amplified by PCR, and the amplicons were purified to remove contaminants. The 8F and 1492R primers were used to carry out the forward and reverse DNA sequencing using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence was generated from forward and reverse sequence data using the aligner software (BioEdit). The 16S region sequence was used to carry out BLAST with the nr database of NCBI Genbank database. Based on the maximum identity scores, the representative sequences were selected for the construction of the phylogenetic tree using MEGA 6.

Hip-TG based profiles of Calothrix sp. culture and plant samples

The cyanobacteria-specific primers HIP-TG (5'- GCGATCACTG- 3') and STRRmod (5'-GCG CCC CAA TCC-3') was used for PCR amplification of DNA from *Calothrix* sp. and plant root/shoot tissues using the method given in Nayak *et al.* (2009). The primers were synthesized by Bangalore Genei (India). The PCR volume was 25 µL, containing 50 pmol of each primer, 2 mM dNTP, 2.5% (v/w) BSA, 10% (v/v) DMSO, 1U of *Taq* DNA polymerase, *Taq* buffer and 5 µL of cyanobacterial filaments. The amplifications were performed in a Peqlab QB96 thermocycler as follows: one cycle at 95°C for 6 min, 35 cycles at 94°C for 1 min, 56°C for 1 min, 65°C for 5 min, and 65°C for 16 min, with the final step at 4°C. The PCR amplification products were resolved on 1.5% agarose gel prepared using TAE (Tris acetate EDTA; 1X) buffer and electrophoresis was done at 80 V for 2 h. The PCR reactions were repeated at least three times to ascertain the reproducibility of the band profiles. The size of bands in the profiles were determined by comparison with the profiles of standard molecular markers (1 kb ladder), procured from Bangalore Genei (India).

Statistical analyses

The SD (Standard deviation) values were calculated using Microsoft Excel and depicted in the graphs as error bar. Analysis of variance (ANOVA) was performed using Windostat 8.5

statistical package, according to the experimental design, arranged as a CRD (completely randomized design), with seven treatments, including control (T1). The factors included the different cultures as treatments, and triplicate sets of data were recorded. The variation among the treatments for the various parameters was analyzed by ANOVA ($P < 0.05$). Correlations were analysed using Pearson's coefficient in the Microsoft Excel package. Standard deviation (SD) is depicted as error bars in the graphs and lower case alphabets denote rankings representing significantly different values among the treatments based on SPSS-16 statistical package.

Results

Evaluation of growth and physiological attributes of *Calothrix elenkinii*

In order to study the growth parameters, the chlorophyll content of strain was estimated at different growth stages (1, 2, 3 and 4 weeks old cultures). The chlorophyll values ranged from 13-19 µg mL^{-1}, and it was found highest at 4 weeks followed by 3 weeks (Table 1). Protein content values ranged from 197.33 to 594.67 µg mL^{-1}, and IAA production values ranged from 0.081 to 3.7 µg mL^{-1} (Table 1). The values of ARA and IAA production (Fig. 2a) were highest in exponential growth (2-3weeks) phase and lowest in stationary phase (4 weeks). ARA values ranged from 2.01-6.72 nmoles of C_2H_4 mL^{-1} h^{-1} (Fig. 2b).

Among all the measured hydrolytic enzymes, chitosanase activity was higher as compared to activity of other enzymes ranging from 0.075 to 0.1 IU mL^{-1} culture. β-1,3-endoglucanase activity ranged from 0.003 to 0.033 IU mL^{-1} culture and CMCase (β-1,4-endoglucanase) activity values were ranging from 0.007 to 0.012 IU mL^{-1}. The activities of all these hydrolytic enzymes were found to be highest in 3 weeks old culture (Table 2).

Experimental set up with rice crop under controlled conditions

Bacterial population in the root and shoot tissues

Analyses were done with 10d old seedlings. Root and shoot tissue extracts were used for plating to determine CFU on different media - Yeast Extract Mannitol Agar (YEMA), Pikovskaya's Agar, Soil Extract Agar (SEA), R2A Agar and Jensen's Agar. Total count on different media was found higher from root tissues than shoot tissues (Table 3). On YEMA plates, the colony counts from inoculated root and shoot samples were 2. 07 x 10^3 and 1.6 x 10^3 respectively, while from control root and shoot samples it was 2.86 x 10^7 and 2.48 x 10^3

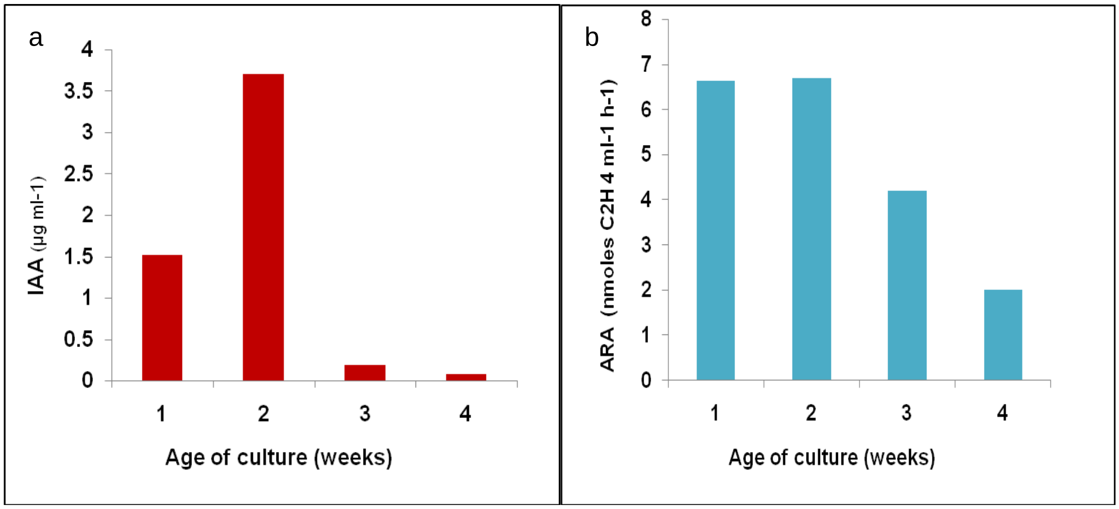


Fig. 2 Physiological attributes of *Calothrix* sp.

a. IAA Production

b. Acetylene Reduction Activity (ARA)

Table 1 Growth and physiological attributes of *Calothrix elenkinii*

Culture age (weeks)	**Chlorophyll ($\mu g\ mL^{-1}$)**	**Proteins ($\mu g\ mL^{-1}$)**
1	13.00 ± 1.00	197.33 ± 9.24
2	12.00 ± 1.00	594.67 ± 16.65
3	17.00 ± 1.04	514.67 ± 20.13
4	19.00 ± 1.06	456.23 ± 16.00
SE(m) ±	0.311	5.150
CD($P \leq 0.05$)	0.862	14.27

[*] All values represent means ± SD; n=3

Table 2 Time course studies on the activity of hydrolytic enzymes in *Calothrix elenkenii*

Culture age (weeks)	**CMCase (β-1,4- glucanase activity) (IU mL^{-1} culture)**	**β-1,3-glucanase activity (IU mL^{-1} culture)**	**Chitosanase activity (IU mL^{-1} culture)**
1	0.007 ± 0.001	0.003 ± 0.001	0.075 ± 0.001
2	0.012 ± 0.001	0.020 ± 0.001	0.080 ± 0.003
3	0.011 ± 0.001	0.033 ± 0.002	0.100 ± 0.001
4	0.008 ± 0.001	0.016 ± 0.001	0.090 ± 0.001
SE(m) ±	0.0001	0.0001	0.0001
CD(***$P \leq 0.05$***)	0.0003	0.0003	0.0003

[*] All values represent means ± SD; n=3

Table 3 Population densities of culturable bacteria from root and shoot tissues of rice plants with and without cyanobacterial inoculation

Media	**Total colony counts (CFU mL^{-1})**			
	Rice plant*		**Rice plant* + *Calothrix***	
	Root	**Shoot**	**Root**	**shoot**
Yeast Extract Mannitol Agar	2.86×10^{7}	2.48×10^{3}	2.07×10^{3}	1.6×10^{3}
Pikovskaya's Agar	3.0×10^{2}	-	4.6×10^{3}	4.5×10^{3}
Soil Extract Agar	2.89×10^{8}	1.87×10^{8}	2.32×10^{9}	1.32×10^{9}
R2A Agar	2.86×10^{8}	2.42×10^{8}	2.44×10^{9}	2.37×10^{9}
Jensen's Agar	6.8×10^{3}	2.5×10^{3}	3.4×10^{2}	3.2×10^{2}
BG-11(N-)	-	-	6.5×10^{2}	1.4×10^{2}
BG-11(N+)	-	-	-	-
N-Free Semi Solid Agar	-	-	-	-

***** 10 d old seedlings were used for analyses respectively. In Pikovskaya medium, the colony counts from inoculated roots and shoots samples were 4.6 x 10^3 and 4.5 x $10^{3,}$ while from control samples (root and shoot) it was 3.0 x 10^2 and zero colony respectively. In SEA plates, the inoculated samples (root and shoot) showing 2.32 x 10^9 and 1.32 x 10^9 CFU respectively, while from control samples (root and shoot) values ranged from 2.89 x 10^8 and 1.87 x 10^8 respectively. In R2A Agar medium, the CFU of inoculated samples (root and shoot) was found to be 2.44 x 10^9 and 2.37 x 10^9 respectively, while from control samples (root and shoot) it was 2.86 x 10^8 and 2.42 x 10^8 respectively. On Jensen's Agar, CFUs from inoculated samples were 3.4 x 10^2 and 3.2 x 10^2 respectively, and from control root and shoot samples, the values were 6.8 x 10^3 and 2.5 x 10^3.

A total of 34 bacterial morphotypes (7 and 9 from inoculated root and shoots respectively and 9 each from root and shoot samples from control) were identified (Table 4), from which 16 distinct morphotypes selected for Fatty Acid Methyl Ester (FAME) analyses (Table 5).

Fatty acid methyl esters (FAME) analysis of culturable microorganisms

The identification of culturable microbial members by the Microbial Identification System (MIS) compared with morphological characteristics and media used for culturing are given in Table 6. These cultural microorganisms represented the shoot or root microbiome of rice plants inoculated with *C. elenkinii*. The media tested included Jensen's agar, Pikovskaya's agar, R2A agar, Soil Extract agar and Yeast Extract Mannitol agar which had varying concentrations of nutrients from the levels of oligotrophy to copiotrophy for microbial growth. The morphological characteristics of these culturable microorganisms were predominantly round colonies, either creamy white or yellow coloured. Using the MIDI Sherlock Microbial Identification system, the FAME analysis for these microorganisms was performed, and the database of aerobic library (RTSBA 6, version 6.21) was used (Fig. 3). When the similarity index value of $\geq$0.5 and the first choice were considered as the criteria for agreement with the library matches, only 60% of the isolates identified was in agreement with the library. In the present analysis, there were two isolates identified with SI values of more than 0.8 while one of isolates could not be identified using this library. Of the two culturable organisms from the Jensen's agar, one had an SI value of more than 0.8, and it was identified as *Bacillus subtilis* (as H1) while another could not be identified using the

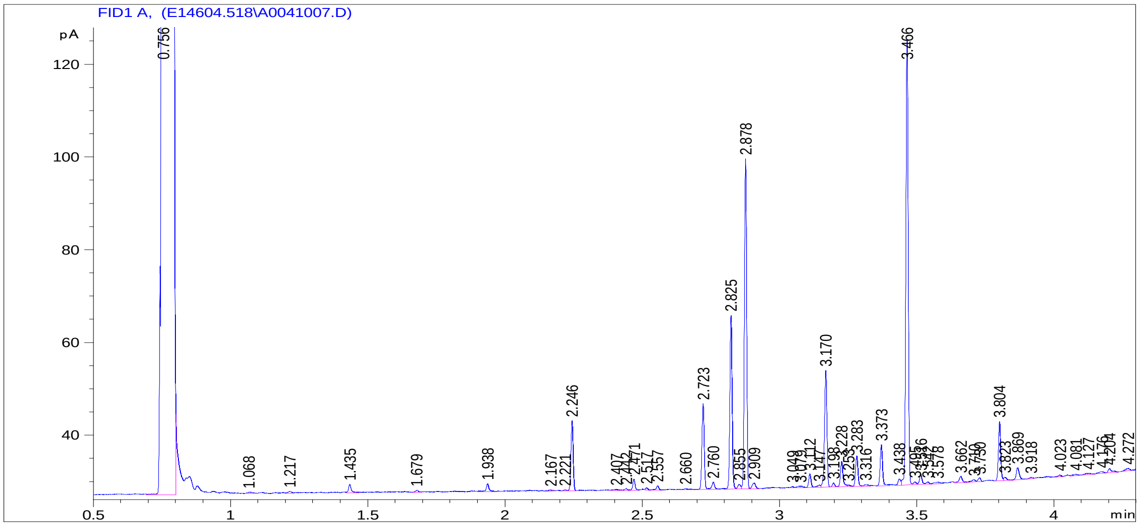


Fig. 3 Fatty Acid Methyl Ester (FAME) profile of a representative of a culturable isolate from the rice microbiome, identified as *Burkholderia cenocepacia* - GC subgroup B (*Pseudomonas cepacia*), with SIM Index of 0.653 using the Library RTSBA6 6.21

Table 4 Morphology of culturable bacteria isolated from different media

S. No.	Sample number	Sample code	Media used	Source	Morphological features of colonies
1	Q1	J1	Jensen's Agar	*Calothrix* shoot	Watery, whitish, mucoid with uneven margin, shiny
2	Q2	J2	Jensen's Agar	*Calothrix* root	Watery, whitish, mucoid with uneven margin, shiny
3	Q3	J5	Jensen's Agar	Control root	Smaller, round with reddish coloured colonies
4	Q4	J6	Jensen's Agar	Control shoot	Smaller white, round
5	Q5	P3	Pikovskaya's Agar	*Calothrix* root	Creamy yellow colored, shiny, round
6	Q6	P4	Pikovskaya's Agar	*Calothrix* shoot	Creamy yellow colored, shiny, round
7	Q7	P6	Pikovskaya's Agar	Control root	Creamy yellow colored, shiny, round
8	Q8	Y1	YEMA	*Calothrix* shoot	Whitish, mucoidal, round
9	Q9	R1	R2A Agar	*Calothrix* shoot	Cream colored, larger sized, round
10	Q10	R2	R2A Agar	*Calothrix* shoot	Yellow colored, pin point small, round
11	Q11	R3	R2A Agar	*Calothrix* shoot	Whitish, small, round
12	Q12	R4	R2A Agar	*Calothrix* root	Creamy white, medium sized round
13	Q13	R10	R2A Agar	Control shoot	White, opaque, round
14	Q14	R11	R2A Agar	Control shoot	Yellow colored, small round
15	Q15	R12	R2A Agar	Control shoot	Cream colored, larger shiny
16	Q16	R13	R2A Agar	Control root	Cream colored, larger sized
17	Q17	S1	Soil Extract Agar	Control root	White, round large sized with uneven margin
18	Q18	S2	Soil Extract Agar	Control root	Cream colored, medium size, uneven margin
19	Q19	S3	Soil Extract Agar	Control root	Watery pink, shiny, smaller round
20	Q20	S4	Soil Extract Agar	Control root	Yellowish white small
21	Q21	S5	Soil Extract Agar	Control root	White round medium sized, with raised surface round
22	Q22	S6	Soil Extract Agar	Control root	Whitish, round with pin point dot in centre medium size
23	Q23	S7	Soil Extract Agar	Control shoot	Cream colored, medium size, highly uneven margin
24	Q24	S8	Soil Extract Agar	Control shoot	White, pin point small round
25	Q25	S9	Soil Extract Agar	Control shoot	Watery pink, shiny, smaller round
26	Q26	S10	Soil Extract Agar	Control shoot	White round
27	Q27	S11	Soil Extract Agar	Control shoot	yellow round, with raised surface round
28	Q28	S18	Soil Extract Agar	*Calothrix* shoot	Watery pink, shiny, smaller round
29	Q29	S19	Soil Extract Agar	*Calothrix* shoot	Whitish, round with dot in centre medium sized
30	Q30	S20	Soil Extract Agar	*Calothrix* shoot	Yellowish white colored, small
31	Q31	S21	Soil Extract Agar	*Calothrix* root	White, with uneven margin
32	Q32	S22	Soil Extract Agar	*Calothrix* root	Cream white colored, medium size, uneven margin
33	Q533	S23	Soil Extract Agar	*Calothrix* root	White, small, round
34	Q34	S24	Soil Extract Agar	*Calothrix* root	Watery pink, shiny, smaller round

Table 5 Details of distinct bacterial morphotypes selected for FAME analysis

S.No	Media	Culture Code	Source	Incubation Temperature	Morphological Characteristics
1	Jensen's Agar	H1	*Calothrix* shoot	37^{o}C	Watery, whitish mucoid with uneven margins shiny colonies
2	Jensen's Agar	H2	Control shoot	37^{o}C	Watery, whitish mucoid with uneven margins shiny colonies
3	Pikovskaya's Agar	H3	*Calothrix* root	30^{o}C	Creamy yellow colored, shiny, round colonies
4	Pikovskaya's Agar	H4	Control root	30^{o}C	Creamy yellow colored, shiny, round colonies
5	R2A Agar	H5	*Calothrix* shoot	30^{o}C	Cream colored, large sized round colonies
6	R2A Agar	H6	*Calothrix* shoot	30^{o}C	Yellow colored pin point small round colonies
7	R2A Agar	H7	*Calothrix* shoot	30^{o}C	Whitish small round colonies
8	R2A Agar	H8	*Calothrix* root	30^{o}C	Creamy white, medium sized round colonies
9	R2A Agar	H9	Control shoot	30^{o}C	Yellow colored small round colonies
10	R2A Agar	H10	Control shoot	30^{o}C	Cream colored large round shiny colonies
11	Soil Extract Agar	H11	Control root	30^{o}C	Cream colored large round shiny colonies
12	Soil Extract Agar	H12	Control shoot	30^{o}C	Watery pink shiny small round colonies
13	Soil Extract Agar	H13	Control shoot	30^{o}C	Cream colored, medium size colonies, eve margin
14	Soil Extract Agar	H14	*Calothrix* shoot	30^{o}C	Whitish round medium sized colony with do centre
15	Soil Extract Agar	H15	*Calothrix* shoot	30^{o}C	Yellowish white colored small colonies
16	YEMA	H16	*Calothrix* shoot	30^{o}C	Watery white mucoid round colony

RTSBA6 aerobic library. Two cultural organisms were selected from the Pikovskaya's medium; one was identified as *Bacillus subtilis* with the SI value of 0.607 and another as *Burkholderia cenocepacia*-GC subgroup B (known earlier as *Pseudomonas cepacia*) with the SI value of 0.653. All the isolates cultured from the R2A agar belonged to the families of Paenibacillaceae, Staphylococcaceae, Bacillaceae, Planococcaceae of the Phylum Firmicutes except one which belonged to the Family Micrococcaceae of the Phylum Actinobacteria. This member of the Family Micrococcaceae was identified as *Arthrobacter oxydans* with the SI value of 0.781. Likewise, all the isolates from the soil extract agar medium belonged to the Family Bacillaceae except an isolate that belonged to the Family Micrococcaceae, and was identified as *Arthrobacter globiformis* GC subgroup with the SI value of 0.756. The species of the genus *Bacillus* from the soil extract agar included *B. pumilus*, *viscosus*, *megaterium* and a member of GC group 22 with no 16S match to known species (having the SI value of 0.420 only). Interestingly, the culturable microorganism isolated from the Yeast Extract Mannitol agar medium belonged to the Phylum Proteobacteria and was identified as *Stenotrophomonas maltophilia* with the SI value of 0.865 (Table 6).

16S rRNA sequencing of isolates and their phylogenetic analyses

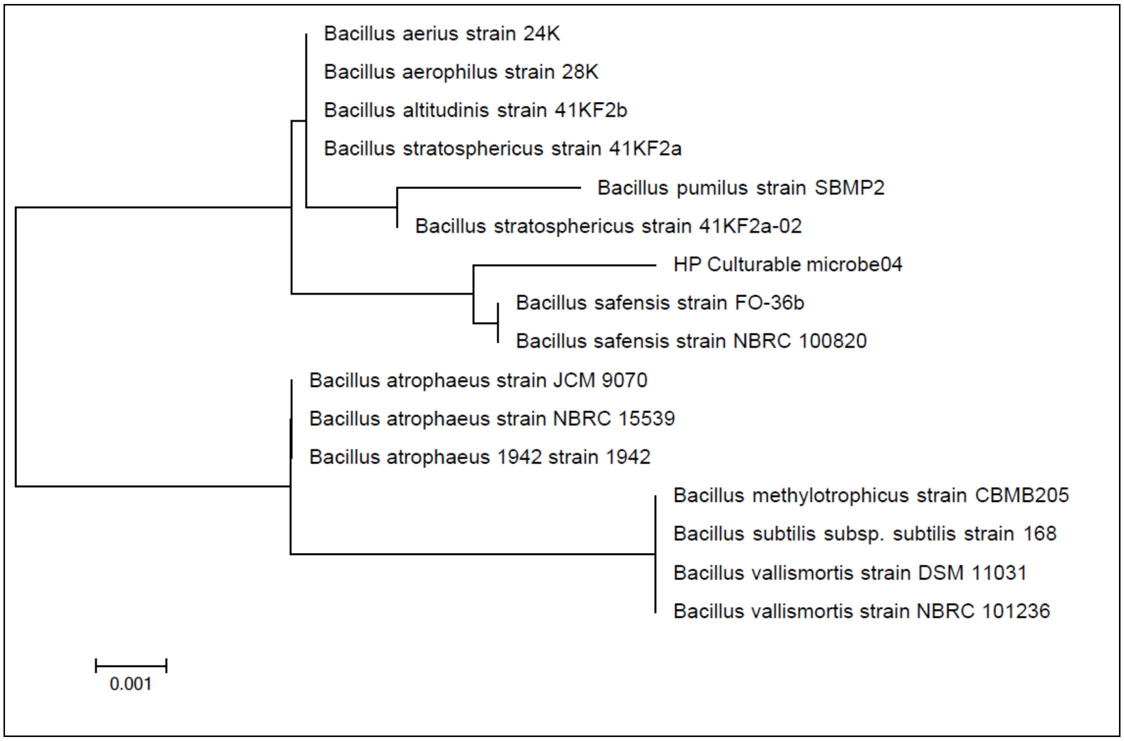
16S rRNA gene sequencing of the culturable microorganisms was done. Phylogenetic relationships between the selected culturable microorganisms and the representatives from the NCBI database were established (Fig. 4-8). The members of Genus Bacilliaceae were found to be predominant. Only one of the six isolates belonged to the Genus Pseudomonadaceae, with the sequences similar to *Pseudomonas plecoglossicida* strain FPC951.

Nitrogenase activity of isolates growing in N free media

Among the five isolates found to grow on nitrogen free media- 4 (J1, J2, J3 J4) from Jensen's medium and one from YEMA medium (Y1), J1 showed very low values. The other four isolates showed values ranging from 3.1 – 3.53 nmoles vial^{-1}h^{-1}.

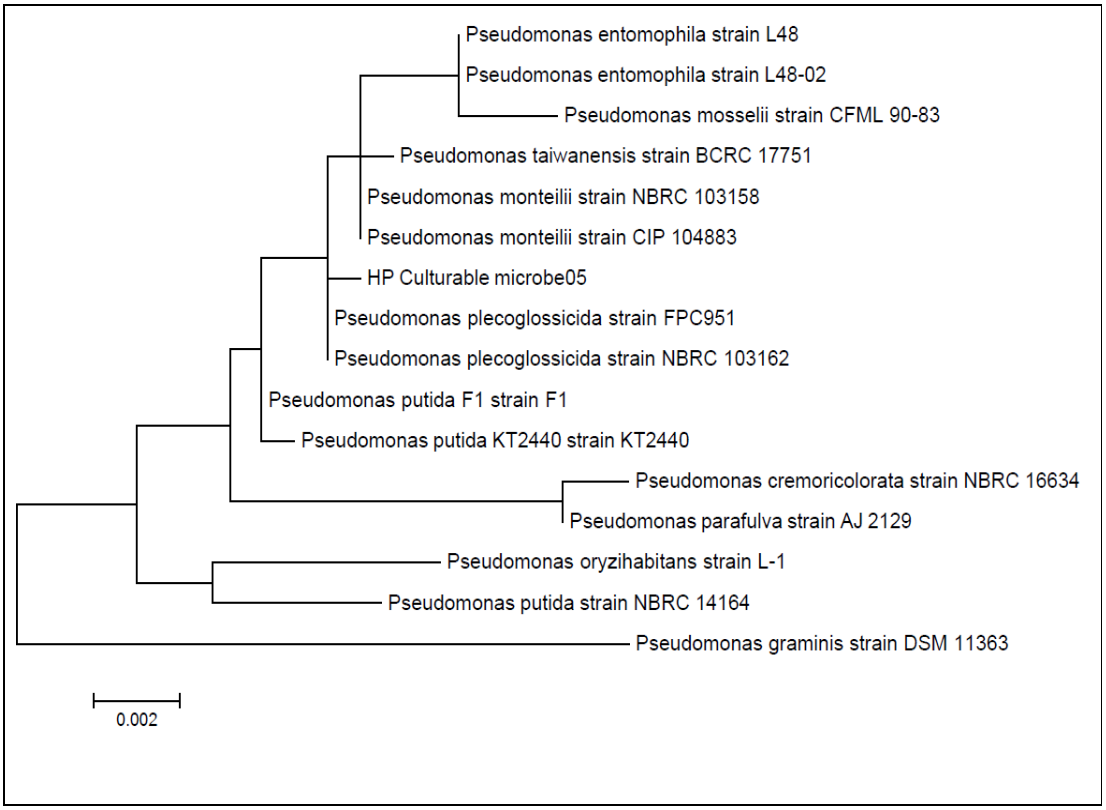
Qualitative and quantitative measurement of P solubilization by isolates

Three isolates showing zone of solubilization ranging from 3.1 to 7.3 mm on Pikovaskaya medium were grown in Pikovaskaya broth for 3 d (Fig. 9). The available P in the filtrates was found to be highest (264.58 ug mL^{-1}) in isolate P3 (Table 7), which also showed a zone of 7.3



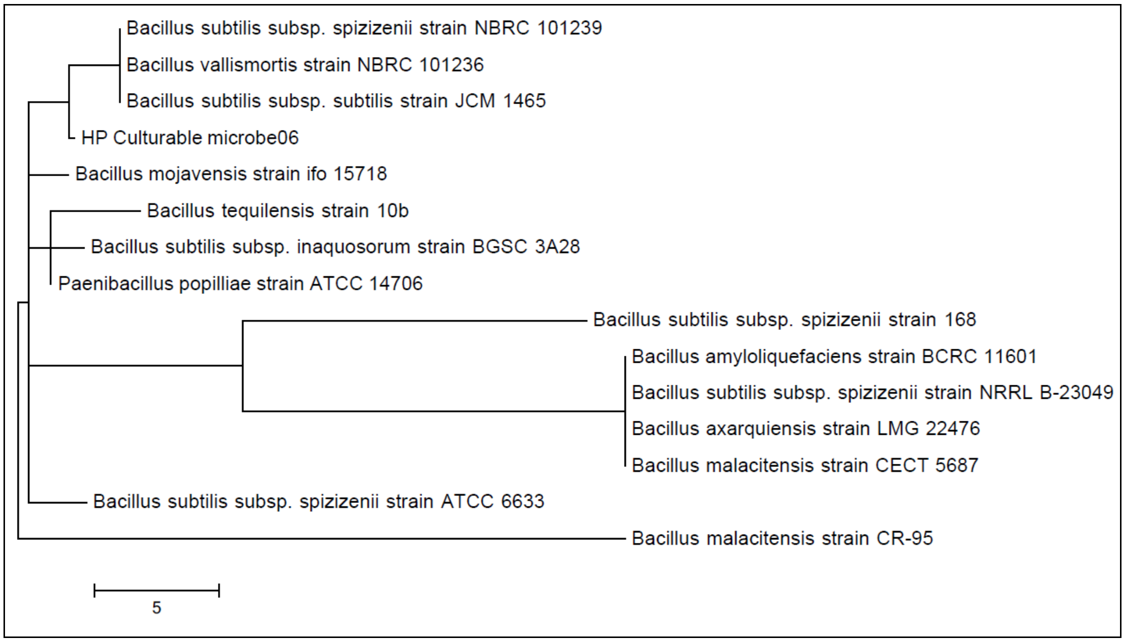
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae

Fig. 4 Phylogenetic analysis of a culturable isolate RM4, from the rice microbiome, by maximum likelihood method using MEGA 6



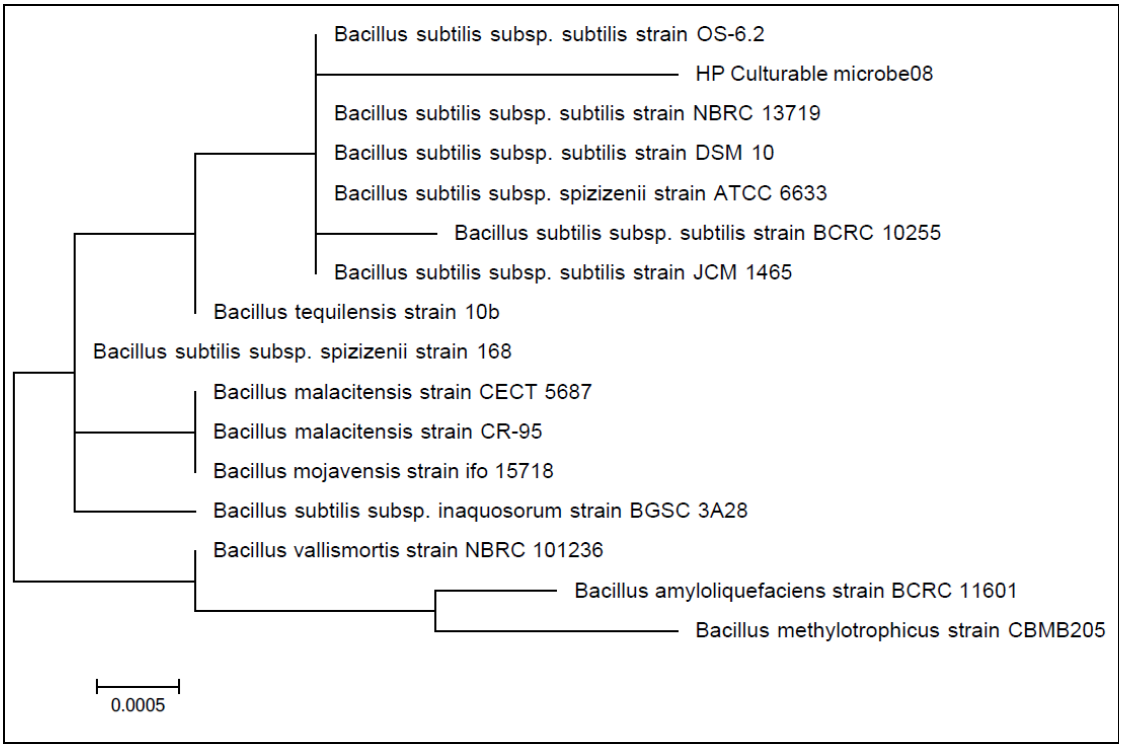
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae

Fig. 5 Phylogenetic analysis of a culturable isolate RM5, from the rice microbiome, by maximum likelihood method using MEGA 6



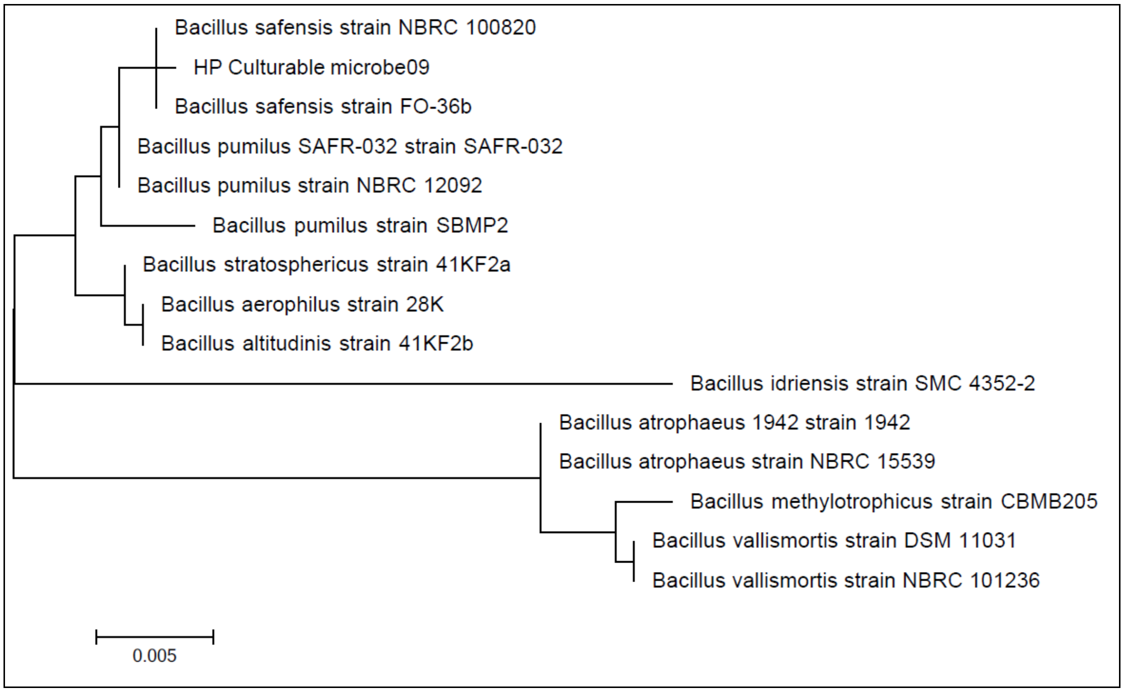
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae

Fig. 6 Phylogenetic analysis of a culturable isolate RM6, from the rice microbiome, by maximum likelihood method using MEGA 6



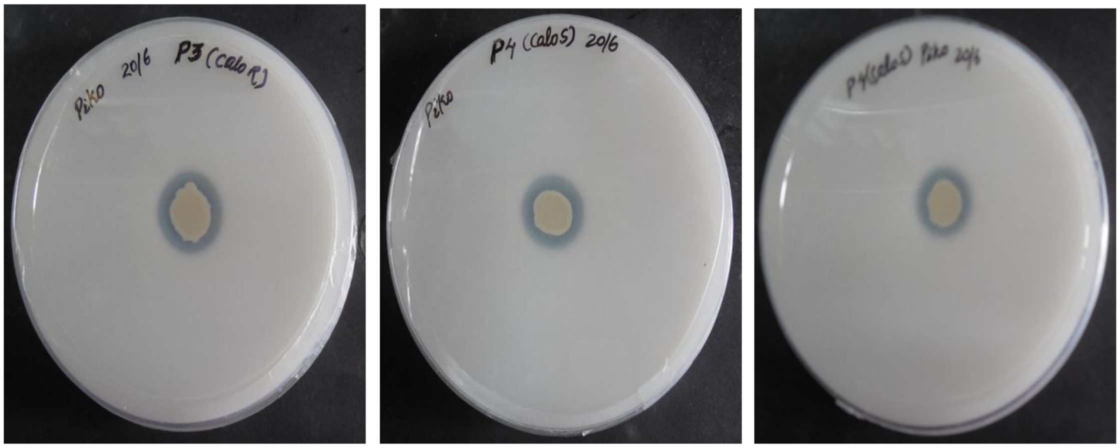
Phylum: **Firmicutes**
Class: **Bacilli**
Order: **Bacillales**
Family: **Bacillaceae**

Fig. 7 Phylogenetic analysis of a culturable isolate RM8, from the rice microbiome, by maximum likelihood method using MEGA 6



Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae

Fig. 8 Phylogenetic analysis of a culturable isolate RM9, from the rice microbiome, by maximum likelihood method using MEGA 6



P3 P4 P6

Fig. 9 Zone of solubilization produced by the isolates from the rice microbiome on Pikovaskaya Agar medium.

Table 6 FAME based identification of selected culturable isolates from the rice microbiome

Cultures	Media	Identity of genera	SIM Index
H1	Jensen's Agar	*Bacillus subtilis*	0.807
H2	Jensen's Agar	No matches found in RTSBA6	-
H3	Pikovskaya's Agar	*Bacillus subtilis*	0.607
H4	Pikovskaya's Agar	*Burkholderia cenocepacia-GC* subgroup B (*Pseudomonas*)	0.653
H5	R2A Agar	*Arthrobacter oxydans*	0.781
H6	R2A Agar	*Paenibacillus validus* (*Bacillus gordonae)*	0.403
H7	R2A Agar	*Staphylococcus hominis hominis*	0.489
H8	R2A Agar	*Bacillus cereus GC* subgroup A	0.665
H9	R2A Agar	*Staphylococcus cohnii cohnii*	0.652
H10	R2A Agar	*Kurthia gibsonii*	0.453
H11	Soil Extract Agar	*Bacillus* GC group 22 (No 16S match to known species)	0.420
H12	Soil Extract Agar	*Bacillus pumilus* GC Subgroup B	0.535
H13	Soil Extract Agar	*Bacillus vicosus* (was *Arthrobacter*)	0.463
H14	Soil Extract Agar	*Arthrobacter globiformis*-GC Subgroup A	0.756
H15	Soil Extract Agar	*Bacillus megaterium*-GC Subgroup A	0.598
H16	Yeast Extract Mannitol Agar	*Stenotrophomonas maltophilia*	0.865

Table 7 Qualitative and quantitative analyses of phosphate solubilization by the isolates from the rice microbiome

Bacterial culture code	**Zone of hydrolysis (mm) (3d incubation)**	**Available P (µg/ml) (3 d incubation)**
P3	7.33 ± 0.15[*]	264.58 ± 0.87
P4	7.07 ± 0.23	237.49 ± 0.64
P6	3.10 ± 0.15	37.53 ± 0.66
SE(m) ±	0.054	0.253
CD($P \leq 0.05$)	0.15	0.70

[*] All values represent means ± SD; n=3

mm. Isolates P3, P4 and P6, showed zones of 7.3, 7.0 and 3.1 mm, and available P of 264.58, 237.5 and 37.5 ug mL^{-1}respectively.

Plant biometric parameters

The root and shoot length of 10d old plants measured revealed an increase of three-four folds in inoculated treatment, as compared to control (Fig.10a-b). The shoot length values of inoculated and control were 3.83 and 16.57 cm respectively. Similarly root length of same was 2.80 and 10.17cm respectively. The fresh weight of plants also measured and it showed that 300% increase in inoculated plant as compared to control. The fresh weight values were 80 mg in control and 320 mg in inoculated treatment. Dry weight values showed 66.7% increase in *Calothrix* inoculated samples as compared to control and values were 15 and 25 mg in control and inoculated respectively. The chlorophyll content of roots and shoots were measured, and it was found that 41.2 and 148.3% increases in inoculated root and shoot tissues were recorded respectively, as compared to control (Table 8). The chlorophyll content values were 3.38 and 14.07 μg g^{-1} fresh tissues in control and inoculated root respectively, while in shoot tissues it was 18.17 and 26.95 μg g^{-1} fresh tissues in a similar trend.

Microbial activity of root and shoot tissues

Indole Acetic Acid (IAA) was estimated from control and inoculated plants, which revealed an increase of 1.42 and 7.76 times in inoculated root and shoot respectively, as compared to control (Table 8). The values of IAA production were 1.87 μg mL^{-1} and 2.66 μg mL^{-1} in control and inoculated root tissues respectively. The values recorded were it was 0.80 μg mL^{-1}and 6.21 μg mL^{-1} in control and inoculated shoot tissues respectively. The ARA of plant samples was estimated, and it was 2.94 and 11.11 μ moles of ethylene plant^{-1} h^{-1} in control and inoculated plants respectively (Table 8). Inoculation with *Calothrix* sp. enhanced the nitrogenase activity approximately 4 folds, as compared to control.

Activity of hydrolytic and plant defense enzymes

The CMCase (β-1,4-endoglucanase) activity in root and shoot tissues was measured, and higher activity in root than in shoot tissues, both in control and inoculated was observed. This enzyme activity was also very high as compared to activity of other enzymes (Table 9). The values of CMCase activity in inoculated root and shoot tissues were 11.57% and 18.60 % more as compared to un-inoculated controls. The enzyme activities in roots and shoots

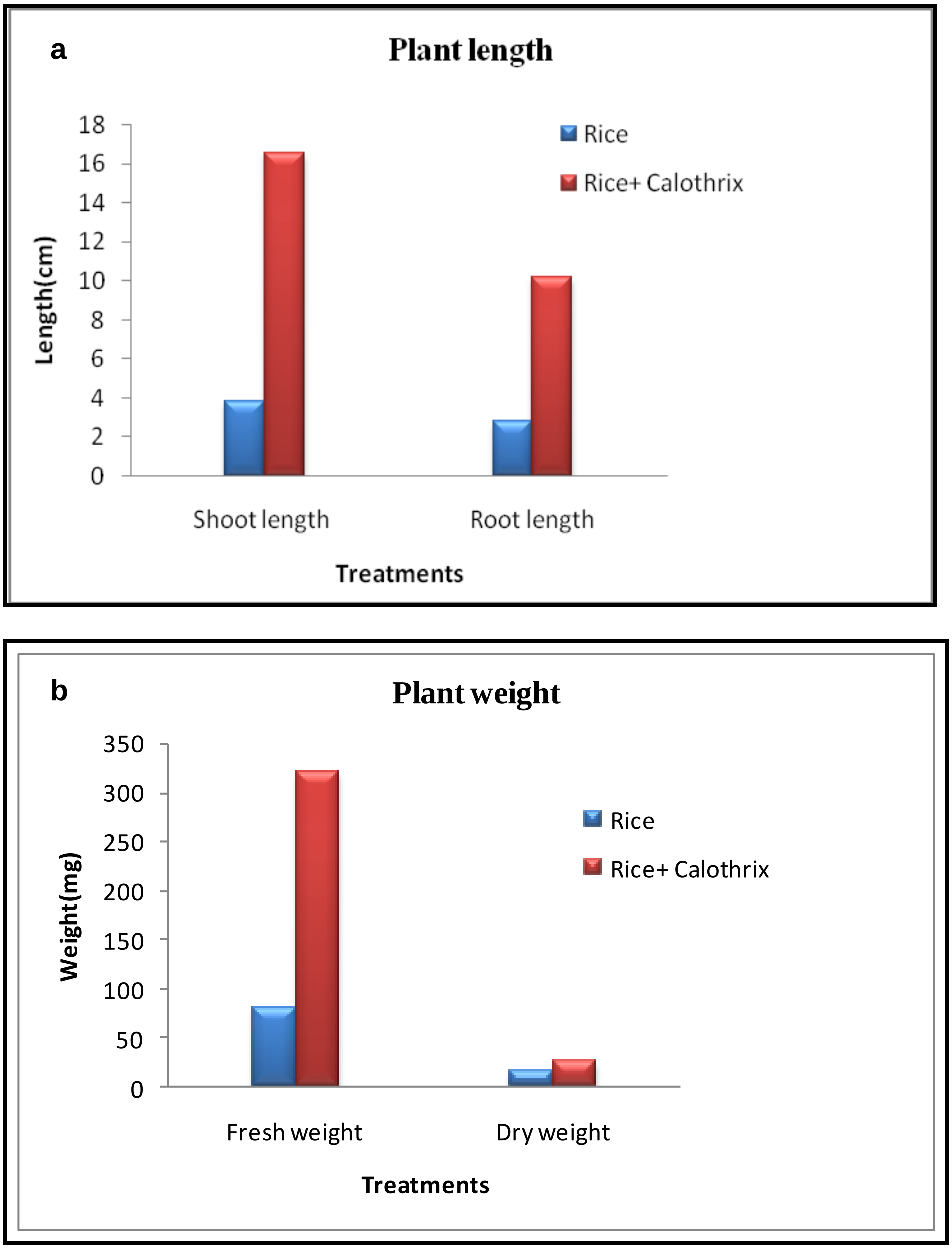


Fig. 10 **Analyses of biometrical parameters of rice seedlings grown with/without cyanobacterial inoculation. a. Length (cm) of roots and shoots; b. Fresh and dry weight (mg)**

Table 8 Estimation of Indole Acetic Acid (IAA) production, chlorophyll content and nitrogenase activity of rice plants[#]

Treatments	**IAA ($\mu g\ mL^{-1}$)**		**Chlorophyll ($\mu g\ g^{-1}$ fresh tissues)**	**ARA (nmoles C_2H_4 $plant^{-1}\ h^{-1}$)**
	Root	**Leaf**	**Leaf**	**Plant**
Rice	1.87 ± 0.08[*]	0.80 ± 0.13	18.17± 0.12	2.94 ± 0.21
Rice + *Calothrix*	2.66 ±0.11	6.21 ± 0.08	26.95 ± 0.55	11.11 ± 0.63
SE(m) ±	0.047	0.033	0.176	0.200
CD($P \leq 0.05$)	0.130	0.091	0.487	0.550

[*] All values represent means ± SD; n=3, [#] 10 d old seedlings were used for analyses

Table 9 Activity of hydrolytic enzymes in rice plants# due to cyanobacterial inoculation

Treatments	**CMCase (β-1,4-glucanase) activity (IU mg^{-1} fresh wt.)**		**Chitosanase activity (IU mg^{-1} fresh weight)**		**Peroxidase activity(PO) (IU g^{-1} fresh weight)**		**Polyphenoloxidase activity (PPO) (IU g^{-1} fresh weight)**		**Phenylalanine ammonia lyase activity(PAL) (IU g^{-1} fresh weight)**	
	Root	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Rice	67.91 ± 0.6*	28.97 ± 1.30	51.00 ± 1.0	34.00 ± 2.0	258.33 ± 12.58*	388.33 ± 7.64	5. 67 ± 1.15	21. 67 ± 2.88	95.54 ± 0.93	188.68 ± 21.2591
Rice + *Calothrix*	75.77 ± 0.7	34.36 ± 0.5	62.00 ± 1.0	43.00 ± 2.0	911.67 ± 27.53	1761.67 ± 62.91	16. 67 ± 0.88	33. 33 ± 2.88	104.39 ± 3.14	205.48 ± 1.2591
SE(m) ±	0.333	0.167	0.289	1.258	6.67	2.21	1.04	0.83	0.64	1.53
CD(P ≤ 0.05)	0.923	0.463	0.801	3.486	18.49	6.12	2.88	2.30	1.81	4.24

* All values represent means ± SD; n=3, # 10 d old seedlings were used for analyses

tissues were 0.068 and 0.076IU g^{-1} fresh weight in control and inoculated root respectively. In shoot tissues, it was 0.029 and 0.035 IU g^{-1}fresh weight. The chitosanase activity in root was more as in compared to shoot tissues and values of activity were 0.051 IU g^{-1} fresh weight and 0.062 IU g^{-1} fresh weight in control and inoculated root tissues, while in shoot tissues it was 0.034 and 0.043 IU g^{-1} fresh weight in control and inoculated respectively.

Plant defense enzyme activity, in terms of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase was analysed in root and shoot tissues samples of the rice seedlings (Table 9). Peroxidase activity in root samples of control and inoculated seedlings was 258.33 and 911.67 IU g^{-1} fresh weight respectively while in shoot tissues it was 388.33 and 1761.67 IU g^{-1} fresh weight respectively. There was 3.53 and 4.53 times increase in enzyme activity in inoculated roots and shoot tissues respectively as compared to un-inoculated control. PPO activity in shoot tissues was more as compared to root tissues in both inoculated and un-inoculated control. The values were 5.67 and 16.67 IU g^{-1} fresh weight in control and inoculated root tissues respectively, while in shoot tissues it was 21.67 and 33.33 IU g^{-1} fresh weight respectively. It was found that there is 2.94 and 1.53 times more activity in the inoculated root and shoot tissues respectively as compared to respective controls. The PAL activity in shoot tissues was higher than in root tissues, and the values were 95.54 and 104.39 IU g^{-1} fresh weight in control and inoculated root tissues, while in shoot tissues it was 188.68 and 205.48 IU g^{-1} fresh weight in control and inoculated samples.

SEM observations of root and shoot sections revealed the presence of rod shaped cells in all the samples (Fig. 11). However, the *Calothrix* inoculated samples exhibited a visible increase in the number of cells and also the presence of short 2-3 celled filaments, presumably of *Calothrix* sp.

Profiling of plant DNA using cyanobacterium specific primers

DNA fingerprinting and analyses of *Calothrix elenkinii* at different growth stages, using two primers- STRRmod and HIP-TG was undertaken (Fig.12a). The profiles revealed that the band sizes ranged from of 1700 bp in STRR1A in all weeks old cultures, 500- 3500 bp in STRRmod, with maximum number of bands in the 500- 3000 bp and 250 -3000 bp in HIP-TG with maximum number of bands in 250- 1800 bp (Fig. 12b). HIP-TG was selected for comparison with the profiles of plant root and shoot with pure culture of *Calothrix elenkinii*

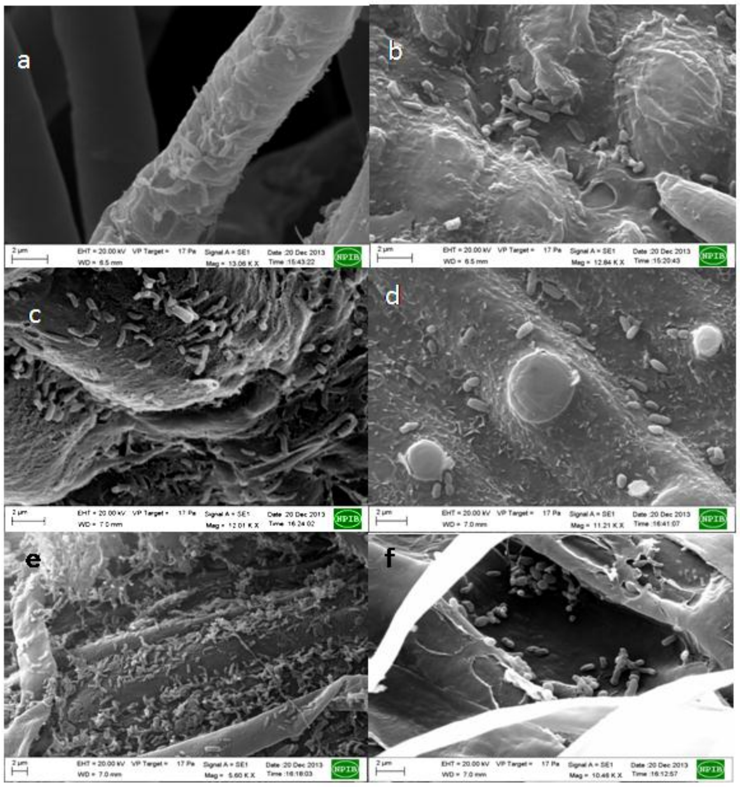


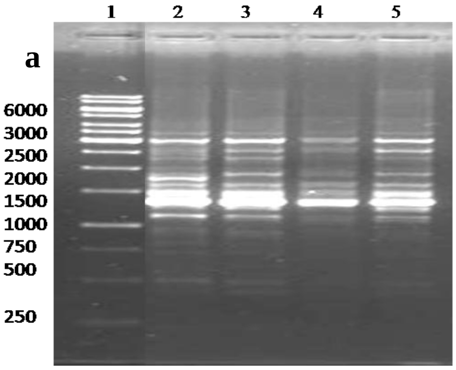
Fig. 11 Scanning electron micrograph showing rod shaped bacterial cells in the sections of rice seedlings. Control seedlings – a., b. root and shoot tissues; Inoculated with *Calothrix* sp. - c., e., f. root; d. shoot tissues.

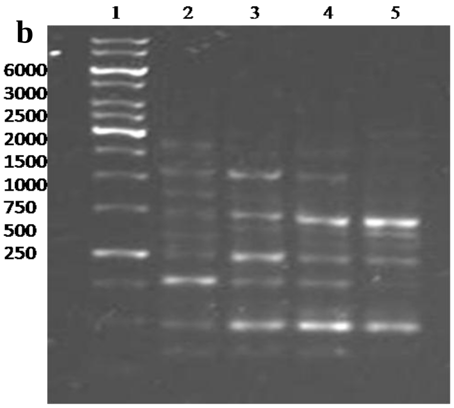
which revealed specific and unique bands, which could be distinguished as those belonging to the inoculated cyanobacterial culture (Fig. 12c). The size of bands ranged from 250 - 4000 bp, with maximum number of bands in the 250-3000 bp region. No amplification was recorded with the DNA fingerprints of root /shoot DNA of control seedlings.

Discussion

Plants and microbes are known to have co-evolved; with extensive communication occurring between plants and microorganisms during different stages of plant growth and development, wherein signaling molecules play an important role (Partida and Hiel 2011; Ortiz-Castro *et al.* 2009). It is well established that the ecological linkages between aboveground and belowground biota represent the main drivers for sustained crop health and crop productivity (Barret *et al.* 2011). Several reports are available on the beneficial effects of plant-associated microbes, such as facilitating nutrient mobilization and uptake, improving plant growth through the production of phytohormones or protecting against phytopathogens (Bais *et al.* 2006; Ali *et al.* 2009; Kandasamy *et al.* 2009). However, the microbiome of rice has been less explored, especially in relation to microbial inoculation.

Among the various inhabitants of rice fields, cyanobacteria play a significant role in providing photosynthetically fixed carbon, nitrogen, besides producing phytohormones and polysaccharides which promote plant growth and soil structure respectively (DE 1939; Mandal et al. 1998). Cyanobacteria exhibit a broad ecological and metabolic diversity, and their structural-functional plasticity confers great versatility, enabling them to adapt and inhabit a wide range of environments and niches, including symbiotic/associative symbioses with different members of the plant Kingdom (Stewart *et al.* 1983; Prasanna *et al.* 2009, 2012). In the present study, the strain *Calothrix elenkinii* was selected as the inoculant for rice seedlings, grown on water and then in pots with sterile/unsterile soil under controlled conditions of the National Phytotron Facility. This cyanobacterial strain is known to produce bioactive metabolites and hydrolytic enzymes, with antifungal properties (Radhakrishnan *et al.* 2009; Manjunath *et al.* 2010, 2011; Natarajan *et al.* 2012, 2013) and has been evaluated for its PGP and biocontrol potential in pot/field studies with different crops (Karthikeyan *et al.* 2007; Kumar *et al.* 2013; Prasanna *et al.* 2014a, b).





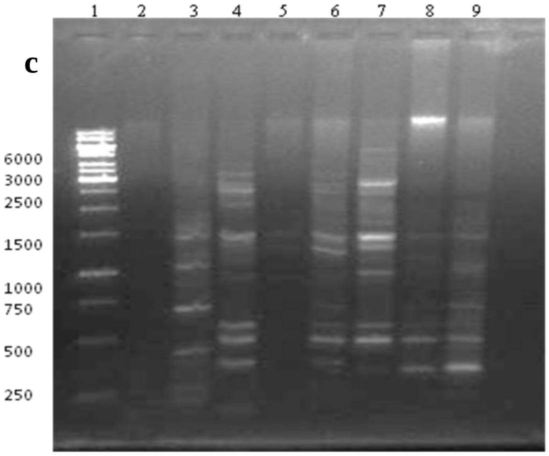


Fig. 12a STRRmod- PCR Fingerprints of the *Calothrix elenkinii* (Lane 1-Marker, 2 to 5- STRRmod, 1, 2, 3, 4W old cultures respectively.

Fig. 12 b HipTG - PCR Fingerprints of the *Calothrix elenkinii* (Lane1-Marker 1kb DNA ladder, 2 to 5- 1, 2, 3, 4 W old cultures respectively)

Fig. 12c HIP-TG PCR based DNA fingerprints of rice seedlings. Lane 1 is the 1 kb marker; lane 2-3 are roots and shoots samples from uninoculated control rice seedlings, lanes 7-8 roots and shoots samples from inoculated rice seedlings; lane 9 is *Calothrix* pure culture.

Artificial cyanobacterial associations have mainly been investigated under controlled laboratory conditions in hydroponics or sand or sterile soil in pot experiments (Svircev *et al.* 1997; Nilsson *et al.* 2002, 2005; Jaiswal *et al.* 2008; Prasanna *et al.* 2009a; Sood *et al.* 2011; Babu *et al.* 2014), and cyanobacterial cells or filaments have been found inter- or intracellularly. The mode of entry is hypothesized to be through the lateral roots or lenticels, mainly mediated through the gliding motility of hormogonia (short filaments of 2-6 cells) in filamentous forms (Nilsson *et al.* 2002).; however, the accompanying biochemical changes have not been investigated, except in terms of plant biomass accretion or nitrogen fixation. In the present study, short two-three celled structures resembling *Calothrix* sp. were observed in SEM. Colonisation on the roots which may also be responsible for the significant enhancement in growth and physiological activity of the seedlings. An interesting observation was the visible enhancement in the number of rod-shaped bacterial cells in *Calothrix* sp. inoculated samples, vis a vis control samples. The increased metabolic activity of the seedlings observed in inoculated plants may be attributed to the selective enhancement of useful flora, which can promote plant growth. This information on the rice plant microbiome can be useful, especially in relation to inoculation with cyanobacteria, which represent a major component of rice field biota, and play a significant role in their sustained fertility of paddy crop (Roger *et al.* 1993).

In the present study, the biometric analysis and biochemical characterization of root and shoot tissues revealed a significant enhancement in plant growth, nitrogenase activity, indole acetic acid (IAA) production and activity of many defense enzymes. This may be attributed to the selective enrichment of useful bacteria and elimination of pathogenic microflora by the colonization of *Calothrix* sp., which is already known to be a promising PGP and biocontrol agent (Kumar *et al.* 2013; Prasanna *et al.* 2014a, b).

Employing cultural approaches, population densities ranging from 10^6-10^9 CFU/mL of culturable microorganisms were recorded in the root and shoot tissues in different types of nutrient media. Cyanobacterial inoculation led to 10 fold higher populations in the different media tested. Functional characterization of these isolates, five from nitrogen-free media (Yeast Extract Mannitol and Jensen’s media) and three isolates from the Pikovskaya medium revealed their potential for nitrogen fixation and phosphorus solubilisation, respectively. The enhanced nitrogen fixing potential of rice seedlings in *Calothrix* sp. inoculated plants can be a net result of selective enrichment of these diazotrphic bacteria.

Plants are also known to cultivate their microbiome and recent reports on model systems such as *Arabidopsis thaliana* grown under controlled conditions in natural soils revealed that host genotype can influence the microbiome (Lundberg *et al.* 2012; Bulgarelli *et al.* 2012). Bulgarelli *et al.* (2012) also observed that soil type defines the composition of root-inhabiting microbial communities, with preferential colonization of *Arabidopsis* roots by members belonging to Proteobacteria, Bacteriodetes and Actinobacteria. Pieffer *et al.* (2013) analysed the diversity and heritability of field grown maize inbreds and provided evidence for heritable variation in the rhizosphere microbial community composition. Although a variety of microbes enter the plant and become transient endophytes, there are several consistent symbiotic or pathogenic candidates. In the present investigation, 34 different morphotypes were selected, among which sixteen morphotypes produced distinct colonies. These were selected for profiling of fatty acid methyl esters (FAME), among which only 60% of the isolates identified were in agreement with the library, when the similarity index value of $\geq$0.5 and the first choice were considered. Two isolates were identified with SI values of more than 0.8 while one of isolates could not be identified using this library. About 50% of the isolates were found to be of the Phylum Firmicutes. Despite the heterogeneity and taxonomic complexity of microbiome known to be present in the shoot and roots of rice, the predominance of the members belonging to the Phylum Firmicutes that are amenable to culturing was evident from the present study. Even though the total number of culturable isolates selected for the FAME analysis and identification was only 16, about 50% of the isolates were found to be of the Phylum Firmicutes. Lucas *et al.* (2014) analysed the structural and functional diversity of rhizosphere of rice and observed the dominant taxon to be Proteobacteria, which represents the largest and metabolically diverse group of soil microbes. Lundberg *et al.* (2012) illustrated that the endophytic compartments of *Arabidopsis thaliana* grown in two geochemically distinct soils are distinct, but show a marked enrichment of Actinobacteria, and specific families from other phyla, notably Proteobacteria.

Most of the reports on rice endophytic bacteria have recorded members belonging to the following groups - *Herbaspirillum seropedicae* (Olivares *et al.* 1996); *Rhizobium* *leguminosarum* (Yanni *et al.* 1997); *Serratia* sp. (Sandhiya *et al.* 2005); *Bradyrhizobium japonicum* (Chantreuil *et al.* 2000*)*; *Klebsiella* sp. (Rosenblueth *et al.* 2004); *Pseudomonas* sp. (You and Zhou 1989); *Azoarcus sp.* (Hurek *et al.* 1994); *Burkholderia* sp. (Engelhard *et al.* 2000); and *Azorhizobium caulinodans* (Engelhard *et al.* 2000). In the present investigation, 16S rDNA sequencing was taken up for selected isolates (from the set of 16 distinct morphotypes), which showed that about 50% of the isolates belonged to the Family Bacillaceae of the Phylum Firmicutes. Although different media with varying concentrations of nutrients were used to isolate the culturable microorganisms, the members of Family Bacilliaceae were found to be predominant. Among the six isolates, only one represented the Family Pseudomonadaceae, with the sequences similar to *Pseudomonas plecoglossicida* strain FPC951. This further emphasizes the pervasive nature of the members of the Bacillaceae and their ability to inhabit diverse environments, such as the plant microbiome, besides extremophilic environments.

Future research should aim at characterizing the key microbial players in the core microbiome of rice and their functions, if any in the functioning of the plant. Also, it would be worthwhile understanding the signaling mechanisms involved, so as to gain a better understanding on the effect of cyanobacterial inoculation on the beneficial and pathogenic microflora in the plant microbiome.

Acknowledgements

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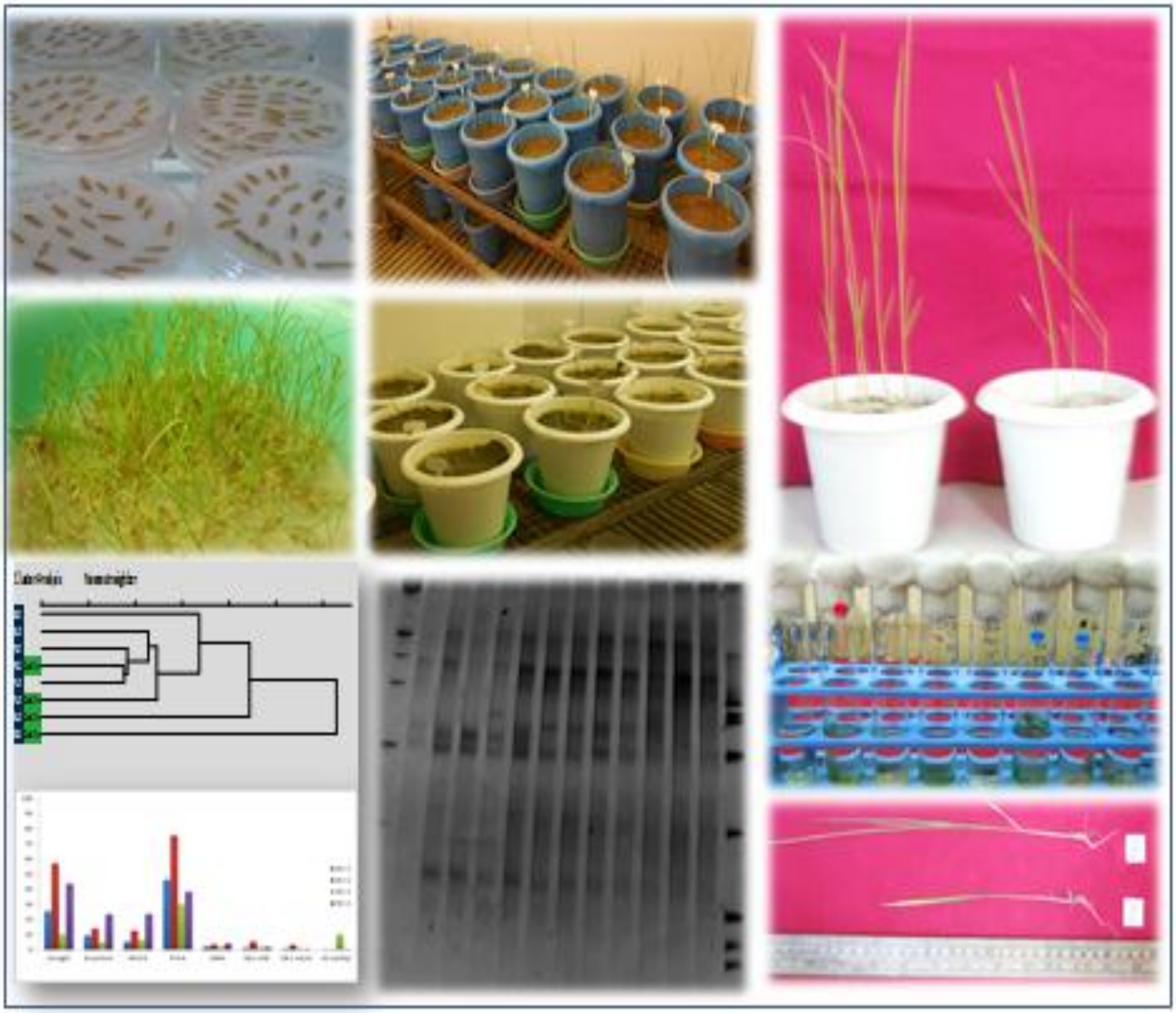


5. RESEARCH PAPER II

5 RESEARCH PAPER II

Profiling of the rice microbiome as influenced by cyanobacterial inoculation

Abstract

Plants can shape their rhizosphere microbiome in order to derive benefits from their interactions with the ubiquitous microorganisms. Cyanobacteria, which are important constituent members on the surface of floodwater and saturated soils, can colonize the roots of rice. The present study was undertaken under controlled environmental conditions, and aimed at monitoring the changes in the microbiome at the community level in different components such as soil, rhizosphere, roots and leaves of rice (cv. Pusa *Sugandh* 5) under the flooded conditions, with or without inoculation of *Calothrix elenkinii*. Growth stage dependent increases in plant biomass (about 70% on dry weight basis compared to control) and nitrogenase activity (about 48%) were observed due to cyanobacterial inoculation. Increases of about 20-50% were observed in the soil chlorophyll content, along with corresponding changes in soil nutrient status. The microbial community profiles were monitored using both sterile and unsterile soil as the growth media by both the DGGE- and PLFA based profiling. While the DGGE community patterns of leaf tissues of plants without inoculation were similar, the patterns of sterile bulk and rhizosphere soil on 45 DAT (days after transplanting) were found to be similar. Increases in total concentrations of PLFAs were by 3 to 16 folds on 45 DAT, relative to the soils on 15 DAT. On 45 DAT, the polyunsaturated fatty acids comprised of 30-40%, followed by straight chain fatty acids (20-29%) and monounsaturated fatty acids (7-18%). The PLFA profiles of soil organisms showed decreased presence of Gram-negative bacteria, anaerobe and Gram-positive bacteria due to cyanobacterial inoculation and provided new evidence on the changes in the bacterial community structure. The present study clearly demonstrated that the cyanobacterial inoculation affected the soil nutrient cycling, more importantly the microbial community structure and plant growth favorably.

Introduction

Rice is a globally important food crop. Extensive information on its complete genome by sequencing and on its proteomics provide valuable insights and better understanding on its responses to abiotic and biotic stress, which represent major challenges in modern agriculture for sustaining and enhancing crop productivity (Lee *et al.*, 2006; Fu *et al.*, 2013). During its cycle from seeds to harvest, these plants interact with microorganisms and derive benefits from their microbial interactions including nitrogen fixation and other nutrient cycling as well as immunity to many plant pathogens. Establishing an adapted symbiosis between agriculturally important crop plants and nitrogen-fixing microorganisms has been much investigated for reducing the dependence on chemical nitrogen fertilizers (Gantar, 2000; Gorelova, 2006). In this context, cyanobacteria are well known to possess the unique capability of forming specific associations with a diverse group of organisms, including protists, animals, Gymnosperms, Pteridophytes and Angiosperms (Babu *et al.*, 2014; Gantar *et al.*, 1991b; Jaiswal *et al.*, 2008; Karthikeyan *et al.* 2009; Prasanna *et al.*, 2009b; Rasmussen *et al*., 1994; Sood *et al.*, 2011). With the advances made in nitrogen fixation, particularly the molecular facets regulating biological nitrogen fixation as well as our knowledge on endophytes and natural diversity among diazotrophs, there is a need to encourage thinking beyond conventional biological nitrogen fixation (Roy and Srivastava, 2013), especially towards developing plants which can be nutritionally independent. In our earlier studies, isolates of *Anabaena* and *Calothrix* formed intimate associations with roots of rice, tomato and wheat plants (Karthikeyan *et al.*, 2009; Prasanna *et al.*, 2009a, 2012a; 2013a). Nilsson and co-workers (2002, 2005) evaluated the competitiveness and colonisation ability of symbiotic cyanobacterial strains, using genetic fingerprints and biochemical studies, however, the chemical signals or plant defense mechanisms were not explored.

Recent advances in research on plant–microbe interactions have revealed that plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host or exhibit characteristic microbial communities when grown on the same soil. Plants are full of microbes and phenotypic expression of most plants in nature is the product of highly co-regulated expression of genes both from plants and microbes (Berendsen *et al*., 2012). Fang *et al.* (2013) illustrated that rice plants can recruit protective microorganisms against pathogens, or modulate allelochemical activity. Several attempts to use the *in situ* hybridization of roots/soil with fluorescently labeled probes and direct microscopy have further provided evidence for the discrepancies observed between culturable microbial diversity and the yet to be cultured or members of the “soil rare biosphere." Lundberg *et al.,* (2012) and Bulgarelli *et al.* (2012) compared the bacterial communities of different *Arabidopsis* genotypes using 454 sequencing of 16S rRNA amplicons at different growth stages and different soil types. They observed a preferential colonization by members belonging to Proteobacteria, Bacteroidetes and Actinobacteria, although some researchers earlier reported a bias for Betaproteobacteria. Evaluation of bacterial microbiota under controlled conditions has revealed a function for metabolically active plant cells and cell wall features in the selection of soil bacteria for host colonization (Bulgarelli *et al.,* 2012). A number of bacteria, especially the Enterobacterial genera such as *Enterobacteria, Serratia, Pseudomonas, Erwinia, Herbaspirillum, Gluconoacetobacter* are known to proliferate in rice rhizosphere and play a significant role in soil and crop productivity (Malik, 2002), but cyanobacteria have been mainly explored to-date as above-ground photosynthetic flora.

Cyanobacteria are an ancient group of photosynthetic prokaryotes, well adapted to a diverse range of environmental conditions and employed as inoculants for enhancing soil fertility and improving soil structure, besides enhancing crop yields, especially in rice (Venkataraman, 1972; Kaushik, 1998; Nayak *et al.,* 2004; Dhar *et al.,* 2007). Later reports have revealed cyanobacteria add organic matter, synthesize and liberate amino acids, vitamins, auxins, reduce oxidizable matter content of the soil, provide oxygen to the submerged rhizosphere, ameliorate salinity, buffer the pH, solubilize phosphates and increase the fertilizer use in various crop plants (Mandal *et al.,*1998; Prasanna *et al.* 2013 a, b,c; 2014b). Earlier reports from IARI are available on the colonization of roots by cyanobacteria in rice, wheat, lignocellulosic substrates and elicitation of defence responses, which indicates numerous interactions of cyanobacteria with the plants (Karthikeyan *et al.*, 2009, Prasanna *et al.* 2009a,b, 2011, 2013a, 2014a,b)

Information on the rice plant microbiome is necessary, especially in relation to inoculation with cyanobacteria, which represent a major component of rice field biota, and play a significant role in their sustained fertility (Roger *et al*., 1993). The questions underlying the present investigation were – can cyanobacteria enter rice plants and if so, what kind of interactions are observed in such associations and to what degree they modulate the microbiome of the soil and host plants through direct or indirect mechanisms. Hence, the major focus was towards analyzing the rice microbiome (microbial communities in the soil, rhizosphere, roots, shoot/ leaves) in the presence and absence of cyanobacterial inoculation, under controlled conditions using sterile and unsterile soil as the growth media. The investigation included the use of DGGE- and PLFA based profiling approaches, complemented by evaluation of soil nutrient status and microbiological parameters.

Materials and Methods

Organisms and growth conditions

The cyanobacterial strain used in the present investigation - *Calothrix elenkinii* (RPC1) strain was obtained from the germplasm of the Division of Microbiology, Indian Agricultural Research Institute, New Delhi. The cyanobacterial strain was purified by standard procedures employing a set of antibiotics (Kaushik 1987). Identification was undertaken using taxonomic keys (Desikachary, 1959) and 16S rDNA sequencing (GenBank Accession No. GU292083; Natarajan *et al.* 2012, 2013). The strain was routinely subcultured and maintained in nitrogen free BG-11 medium (Stanier et al. 1971) and grown under light: dark cycle (16:8 hours) under white light 50-55 µE photons $m^{-2}s^{-1}$ and 28 ± 2°C.

Experimental setup for microbiome analyses

Preliminary experiment using water agar as growth matrix

Seeds of rice (*Oryza sativa* var. Pusa Sugandh 5) were surface sterilized with 0.1% mercury (II) chloride for 1 min and thereafter washed by 70% ethanol for 30 sec and thoroughly washed by sterile water. The seeds were imbibed overnight in sterile water in dark, under room temperature and kept for germination on blotting sheets for 48h. The germinated seeds were placed in glass trays containing sterilized 0.8% water agar. The culture of *Calothrix elenkinii* grown in BG-11-N medium was centrifuged at 10,000 rpm for 10 min. The chlorophyll concentration was determined and the culture with chlorophyll concentration of 5.0 µg mL^{-1} was used for inoculation on to water agar. In the control, no inoculum but an equivalent amount of water was used. The lower portion of trays and beaker was covered with aluminum foil to provide simulated conditions of soil environment. The entire setup comprising two treatments (with or without cyanobacterial culture) was kept in growth chamber, maintained at $27\pm2^{o}C$ and illumination of 50-55 µmol photons $m^{-2}s^{-2}$ light intensity (16/8 h; light/dark cycles). All the treatments were carried out in triplicates.

Experimental setup with soil under controlled conditions

The rice cultivar (cv. Pusa Sugandh-5 (Pusa 2511) was collected from the Division of Genetics, IARI, New Delhi. The soil samples for the pot experiments were collected the experimental fields of IARI, air-dried and processed before use. The soil was of sandy loam type with pH, and electrical conductivity (EC 948 µS $cm^{-1;}$ pH 7.1). Pot experiments were carried out in the controlled environmental chambers of the National Phytotron Facility, IARI, New Delhi. Pots of 10” size were filled with soil samples (8 kg). For the experiment with soil under sterile conditions, a set of pots were autoclaved at 1.05 kg cm^{-2} pressure and 121 °C temperature for one hour on three consecutive days. Another set of pots with unsterile soil was also maintained in the environmental chamber.

Co-culturing of rice seedlings with cyanobacterial strain in pots

Rice seedlings of 2-week old were used for transplantation at five seedlings per pot. For the inoculation with *Calothrix elenkinii,* the cyanobacterial culture with the chlorophyll concentration of 0.89 µg g^{-1} was added to both the sets of sterile and unsterile pots. The treatments used under the study were:

Sterile soil: T1- Rice Plant (control)
T2- Rice Plant + *Calothrix elenkinii*
Unsterile soil**:** T1- Rice Plant (control)

T2- Rice Plant + *Calothrix elenkinii*

A total of 10 pots were maintained for each treatment. The rice plants were grown under controlled environmental conditions (30 °C/25 °C with light: dark cycle (i.e., day/night of 14/10 h), relative humidity maintained at 70% throughout the growth period).

Soil and plant analyses

Both soil (bulk- and rhizospheric) and plants were sampled at 15 and 45 DAT (Days after Transplanting) for soil-, plant biometric-, and molecular microbial analyses. All the samples were collected in triplicates.

Nutrient analysis of soil:

Different available nutrients in soil was estimated by using the following standard methods: (i) estimation of Available N in soil by alkaline permanganate ($KMnO_4$) **(**Subbiah and Asija, 1956), (ii) estimation of available phosphorus (P) in soil (P kg ha^{-1}) (Olsen et al., 1952); and (iii) estimation of organic carbon (%) in soil (Walkley and Black, 1934). The soil pH (1:2.5, soil:water) and EC were measured using Elico pH meter (Piper, 1950).

Soil chlorophyll

Soil chlorophyll was assayed using fresh soil cores, collected with the help of tube augar (from 0-20 cm depth), which was placed in 55 mL glass test tubes (Nayak *et al.*, 2004) Acetone: DMSO (1:1) mixture (10mL) was added to soil at a rate of 4 mL g^{-1} soil and tightly sealed using subaseal stoppers. The contents were thoroughly shaken and the tubes were incubated in dark at room temperature for 48-96 hours, until all the pigments get extracted. Intermittent shaking is done after every 24 h to extract chlorophyll completely. If 10 mL acetone: DMSO mixture is consumed after 24 h then more 10 mL mixture is added to the soil of the tubes. The colored solvent is removed, centrifuged to remove debris and optical density values were taken at 663, 645 and 630 nm, using solvent mixture as control. The calculations were done by using formula as given below:

Soil chlorophyll ($\mu g\ g^{-1}$) = $11.64\ (OD_{663}) - 2.16\ (OD_{645}) + 0.10\ (OD_{630})$

Acetylene reduction assay for soil cores

Acetylene reducing activity (ARA) in soil cores incubated in an atmosphere, containing 10% acetylene, was estimated using gas chromatography of ethylene formed (as an index of nitrogenase activity) and expressed as acetylene reducing activity (ARA). Commercially available standard ethylene was utilized for quantification, and vials with an equivalent volume of water served as controls. 50 mL glass tubes filled with soil (approx. 30 g), were incubated under a gas mixture which had been substituted with 10 % acetylene under standard growth conditions for 1 and 1/2 h. One mL aliquots of gaseous phase were removed and injected into a preconditioned gas chromatograph, housing a two meter long Porapak R stainless steel column and a flame ionization detector. The column temperature was maintained at 100°C and injector and detector at 110°C. A flow rate of 35 mL min^{-1} of N_2 served as the carrier gas. Standard ethylene gas was used for calibration and calculations. All values presented are the means of triplicate measurements and expressed as n moles ethylene produced g^{-1} soil h^{-1} **(**Prasanna *et al.* 2003).

Microbial community analyses

Isolation of genomic DNA for DGGE profiling

Plant root- and shoot samples of each treatment were extracted for DNA by using Ultraclean Plant DNA isolation kit (MoBio Inc, USA). Rice plants were rinsed in sterile distilled water to remove soil particles and then the root and shoot portions were separated. The shoot and root plant samples (0.5 g) were crushed in sterilized mortar-pestle using sterile water and the processed tissues were used for extracting the DNA as per the manufacturer's instructions. Samples from bulk soil were set up as enrichment cultures in different media- Yeast Extract Mannitol Broth and Pikovaskaya Broth at different (10^1 and 10^6) and used for DNA extraction after one week using ZR Fungal / Bacterial DNA Miniprep Kit (Zymo Research Corp., USA). The mixtures of selected cyanobacterial strains available from the germplasm of the Division of Microbiology, IARI, New Delhi were also used as additional reference samples for DGGE profiling.

Amplification of 16SrRNA using degenerate bacterial primers for DGGE

The 16S rRNA PCR fragments were amplified using specific primers 341F with GC-clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTC CCG CCG CCC CCGCCC GCC

TACGGGAGGCAGCAG-3’) and 907R (5’-CCG TCA ATT CMT TTG AGT TT-3’) (Muyzer *et al.*, 1993). The reaction mixture contained 1× Taq buffer, 2.5 mM $MgCl_{2,}$ 0.3mM, each of the deoxynucleotide triphosphate, 10 pmol of each primer, 1U *Taq* DNA polymerase, 50 ng of template DNA and MilliQ to give final volume of 25 µL. The reaction mixture were incubated for amplification in a PEQlab Primus 96 Thermal cycler using the optimized program as described by (Hisbergues *et al.*, 2003). The PCR conditions consisted of initial denaturation of 94°C for 2min., 35 cycles of 94°C (30 sec.), 59°C (1 min.), and 72°C (2 min.), plus one additional cycle with a final 8 min. chain elongation at 72°C.The PCR amplification and agarose gel electrophoresis were performed according to standard procedures (Sambrook *et al.*, 1989). The PCR products were analyzed in horizontal 1.2% (w/v) agarose gel (0.5 mg ethidium bromide mL^{-1}) in TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0). The molecular weight standard was a 1kbp ladder (Fermentas). The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California). The PCR products were stored at20°Cbefore DGGE analysis.

Analyses of PCR products by denaturing gradient gel electrophoresis (DGGE)

All reagents were prepared as described in the Bio-Rad D Gene Instruction Manual and Applications Guide (Bio-Rad, Hercules, CA). The DGGE gels were run using a Bio-Rad DCodeTM Universal Mutation Detection System (Bio-Rad, Hercules, CA). Reagents used were 40% Acrylamide/Bis (37.5:1) (acrylamide, 38.93g, Bis-acrylamide, 1.07g mixed with 100mL of deionized water), which was filtered through 0.45µm filter and stored at 4^oC. For the 50 × TAE Buffer (pH 8.0), Tris base (242 g), glacial acetic acid (57.1 mL), and EDTA (0.5M) were added to 1000mL of deionized water. Then the mixture was autoclaved for 20-30 min. and stored at room temperature before used. The gels used for DGGE were 6% polyacrylamide gel (40%-acrylamide and N,N-methylene bisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1× TAE) containing a linear gradient of the denaturant concentration ranging from 30% to 70% with 1 mm thick. The denaturing gradient gel was run for 6 h at 60^oC and 150 V. After completion of electrophoresis, the gels were stained in an ethidium bromide solution

(0.5mg mL^{-1}).The gel images were recorded using a CCD Video camera attached to AlphaImager 1220 (Alpha Innotech Corporation, California).

Analysis of bulk- and rhizosphere soil PLFAs

The standard protocols of Zelles and Bai (1993) and Buyer *et al.* (2010) were followed for the PLFA analysis. Briefly, the lipid fractions were extracted from 5 g of fresh soil using a modified Blight-Dyer extraction with 19 mL of extractant. After evaporation under a stream of nitrogen, lipids were separated on a solid-phase extraction column. Later, these fractions were eluted with 5 mL of methanol. After evaporation under nitrogen, the phospholipids were transesterified to fatty acid methyl esters, extracted with hexane, evaporated and analyzed by gas chromatography GC, equipped with an autosampler, and flame ionization detector. Fatty acid methyl esters (FAMEs) were separated on a 25 m long × 0.2 mm internal diameter × 0.33 µm film thickness. Initial oven temperature was 190 ^{o}C, ramping to 285 ^{o}C at 10 ^{o}C/min and then to 310 ^{o}C at 60 ^{o}C/min, followed by a hold at 310 ^{o}C for 2 min. Injector temperature was 250 ^{o}C and detector temperature was 300 ^{o}C. The MIS Sherlock (MIDI, Inc, Newark, DE, USA) was used to control the system and FAMEs were identified using the MIDI PLFAD1 calibration mix and naming table. An internal standard of methyl nonadecanoate allowed the calculation of FAME concentrations.

Different fatty acid types which were obtained by the conversion of raw fatty acid data by the Sherlock software included straight, branched, hydroxyl, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), dimethyl acetal and other mixed functional groups. Total PLFAs of different fatty acids were calculated using both absolute (nmole g^{-1} soil) and relative abundances (mole percent of $PLFA_{Total}$). In addition, these fatty acids were also summed into different ‘Biomarker groups’ as suggested by Frostegard and Baath (1996), Zelles (1999) and Ringelberg *et al.* (1997). The summed masses of PLFAs typical of Gram-positive-, and Gram-negative bacteria, actinobacteria, anaerobe, fungi and eukaryotes were also calculated for quantifying both absolute and relative abundances.

Statistical analyses

The SD (Standard deviation) values were calculated using Microsoft Excel and depicted in the graphs as error bar. Analysis of variance (ANOVA) was performed using Windostat 8.5 statistical package, according to the experimental design, arranged as a CRD (completely randomized design), with seven treatments, including control (T1). The factors included the different cultures as treatments and triplicate sets of data were recorded. The variation among the treatments for the various parameters was analyzed by ANOVA ($P < 0.05$). Correlations were analysed using Pearson's coefficient in the Microsoft Excel package. Standard deviation (SD) is depicted as error bars in the graphs and lower case alphabets denote rankings representing significantly different values among the treatments based on SPSS-16 statistical package. The PLFAs (molar %) were analyzed using the principal component analysis (PCA) option in the statistical analysis package of Numerical Dynamics. For the analysis of bacterial community patterns, the presence or absence of distinct and reproducible bands in each of the individual DGGE profiles was converted into binary data, and the pooled binary data were used to construct a composite dendrogram. The software NTSYSpc version 2.02i (Rohlf 1995) of Applied Biostatistics Inc., New York was used to calculate the Jaccard distance index (Jaccard 1908) and to construct the dendrogram using the unweighted pair-group method with arithmetic average (UPGMA).

Results

Experimental setup with soil under controlled conditions

Plant biometric parameters

Growth parameters such as plant length, fresh weight and dry weight of plant samples were measured on two stages (15 and 45 DAT) under sterile soil condition. Since the number of healthy plants was too in low in unsterile soil treatments, sampling was restricted only to 15 DAT. Plant length in uninoculated sterile and unsterile conditions was 27.5 and 25.1 cm respectively, while in *Calothrix* inoculated treatment, it was 33.1 and 30.6 cm respectively (Table 1). Plant length recorded at 15 DAT revealed a 20.36 and 21.91% increase in *Calothrix* inoculated as compared to uninoculated treatments with sterile and unsterile soil respectively. Plant length was relatively higher in sterile soil, as compared to unsterile soil, irrespective of inoculation. At 45 DAT, only sterile

Table 1 Influence of cyanobacterial inoculation on plant length of rice seedlings at 15 and 45 DAT

Treatments	**Plant length (cm)**		
	15 DAT		45 DAT
	Sterile soil	Unsterile soil	Sterile soil
Rice plant	27.51 ± 1.5*	25.10 ± 1.1	49.17 ± 0.29
Rice plant + *Calothrix*	33.12 ± 0.7	30.62 ± 0.4	57.00 ± 2.65
SE(m) ±	0.33	0.27	0.82
CD($P \leq 0.05$)	0.91	0.75	2.27

* All values represent means ± SD; n=3

soil treatments were analyzed. The height of plants in *Calothrix* inoculated treatment recorded 15.9% increase as compared to uninoculated plants, with values of 57.00 and 49 cm respectively.

The fresh weight of plant samples was measured and values revealed an increase of 30.2% and 20.2 % in fresh weight in inoculated as compared to uninoculated treatments in sterile and unsterile soils respectively (Table 2). Fresh weight of plant samples in sterile and unsterile soil was 139 and 109 mg respectively and increased to 181 and 131 mg in *Calothrix* inoculated treatments, respectively. Dry weight of plant samples in uninoculated sterile and unsterile soil was 33 and 23 mg respectively while in inoculated ones it was 42 and 31 mg respectively. An increase of 27.3 and 34.78% increase in inoculated sterile and unsterile soils over the uninoculated treatment was observed. A significant increase in fresh weight (75.36%) in *Calothrix* inoculated treatment was recorded. Fresh weight values of plants in inoculated and uninoculated treatments were 1.21 and 0.69 g respectively. Similarly, dry weight of inoculated plants was 76.47% more as compared to uninoculated treatment, with values of 0.30 and 0.17 g respectively.

Soil nutrient status

The soil used in pot experiment was evaluated for available N, P, Organic carbon at 15 and 45 DAT (Table 3). The values of these nutrients at the beginning of the experiment were also recorded. At 15 DAT, the values of available N showed a significant dip, before recording 40-50% increase at 45 DAT. The *Calothrix* inoculated treatment samples recorded increase from 41.0 to 161.0 mg kg^{-1} soil in 15 and 45 DAT, as compared to the initial (0 d) value of 105.0 mg kg^{-1}. In the case of unsterile soil samples, available N values varied from 59 to 98 mg kg^{-1} soil in uninoculated treatment, and 67 mg kg^{-1} soil in *Calothrix* inoculated treatment.

The available P in soil samples showed highest values in sterile uninoculated samples at 45 DAT (7.0 mg kg^{-1} soil), followed by unsterile uninoculated sample containing 7 mg kg^{-1} soil. The lowest values were recorded in sterile uninoculated samples in the beginning (0 d) (5.0 mg kg^{-1} soil). The available organic carbon values ranged from 0.34 to 1.11 %. Highest values were recorded in sterile uninoculated

Table 2 Analyses of plant fresh weight and dry weight of rice seedlings, as influenced by cyanobacterial inoculation at 15 and 45 DAT

	Fresh weight (mg)			**Dry weight (mg)**		
Treatments	**15 DAT**		**45DAT**	**15 DAT**		**45 DAT**
	Sterile soil	Unsterile soil	Sterile soil	Sterile soil	Unsterile soil	Sterile soil
Rice plant	139.32 ± 1.00[*]	109 .31± 1.00	690.06 ± 66.58	33.37 ± 1.00	23.26 ± 1.00	170.39 ± 11.54
Rice plant + *Calothrix*	181.24 ± 1.00	131.04 ± 1.00	1210.37 ± 60.27	42.24 ± 1.00	31.64 ± 1.00	300.51 ± 15.27
SE(m) ±	1.00	1.00	22.00	1.00	1.00	3.00
CD($P \leq 0.05$)	2.00	2.00	60.00	2.00	2.00	8.00

[*] All values represent means ± SD; n=3

Table 3 Macronutrient (Available N, P and Organic carbon) analysis of bulk soil samples at 0,15 and 45 DAT

Soil	**Treatments**	**Available Nitrogen ($g\ kg^{-1}$)**			**Available Phosphorus ($g\ kg^{-1}$)**			**Organic carbon (%)**		
		0 DAT	15 DAT	45 DAT	0 DAT	15 DAT	45 DAT	0 DAT	15 DAT	45 DAT
Sterile Soil	Rice plant	105.21 ± 0.85	48.25 ± 1.06	137 ± 1.61	5.05 ± 0.04	1.19 ± 0.09	7.44± 0.38	0.89 ± 0.035	0.45 ± 0.002	1.11 ± 0.103
	Rice plant + *Calothrix*	105.21 ± 0.85	41.35 ± 0.55	161 ± 1.38	5.05 ± 0.04	1.19 ± 0.13	6.84 ± 0.34	0.89 ± 0.035	0.47 ± 0.004	0.77 ± 0.006
Unsterile Soil	Rice plant	9830 ± 0.59	59.32 ±0.60	ND*	6.55 ± 0.30	1.19 ± 0.04	ND	0.39 ± 0.005	0.35 ± 0.005	ND
	Rice plant + *Calothrix*	98.30 ± 0.59	67.24 ± 1.20	ND	6.55 ± 0.30	1.19 ± 0.04	ND	0.39 ± 0.005	0.34 ± 0.002	ND

*ND- Not detected

samples (1.11 at 45 DAT from 0.89 %). As compared to 15 DAT, the samples from 45 DAT exhibited more organic carbon.

Soil ARA and chlorophyll estimation

Soil ARA and chlorophyll were measured in 15 DAT samples (Table 4). ARA values ranged from 25.4 to 37.6 nmoles ethylene g^{-1} h^{-1}. Highest ARA values were recorded in unsterile inoculated soil samples (37.6 nmoles ethylene g^{-1} h^{-1}), while lowest values were recorded in sterile uninoculated soil samples (25.4 nmoles ethylene g^{-1} h^{-1}).

Evaluation of soil chlorophyll at 15 DAT revealed that highest values (3.19 mg g^{-1} soil) were observed in sterile inoculated soil samples The chlorophyll content in sterile and unsterile uninoculated soils were 2.05 and 1.63 mg g^{-1} soil respectively, while in inoculated soil samples it was 2.19 and 1.96 mg g^{-1} soil respectively. An increase of 20-50% increase was recorded, as a result of inoculation.

Characterization of microbial communities

DGGE profiling

The diversity of bacterial communities in different enrichments using soils from the experiment on the influence of cyanobacterial inoculation was assessed using the culture-independent PCR-DGGE technique. The bands of DGGE profile, consisting of few and very intense bands are likely to be associated with distinct bacterial species. Fig. 1 shows the DGGE profile of enrichments and the mixtures of different cyanobacteria. Both the number of bands and intensities of individual bands showed remarkable changes. Many extra bands and of slightly different intensities were present within the lanes of the mixtures of cyanobacteria. The total number of bands from various enrichments and the cyanobacterial mixtures was limited in this DGGE pattern. The cluster analysis using UPGMA method showed that the enrichments using the Pikovskaya’s medium and all the cyanobacterial mixtures clustered together, suggesting similar community structure (Fig. 2). Similarly, the bacterial community structures of enrichments using Yeast Extract Mannitol Broth showed a higher degree of similarity except that of enrichment using sterile soil with cyanobacterial inoculation. When the DGGE profiles of leaf/shoot, root of rice plants grown using water agar, with or without *Calothrix elenkinii* were analysed, the bacterial communities of leaf/shoot and root

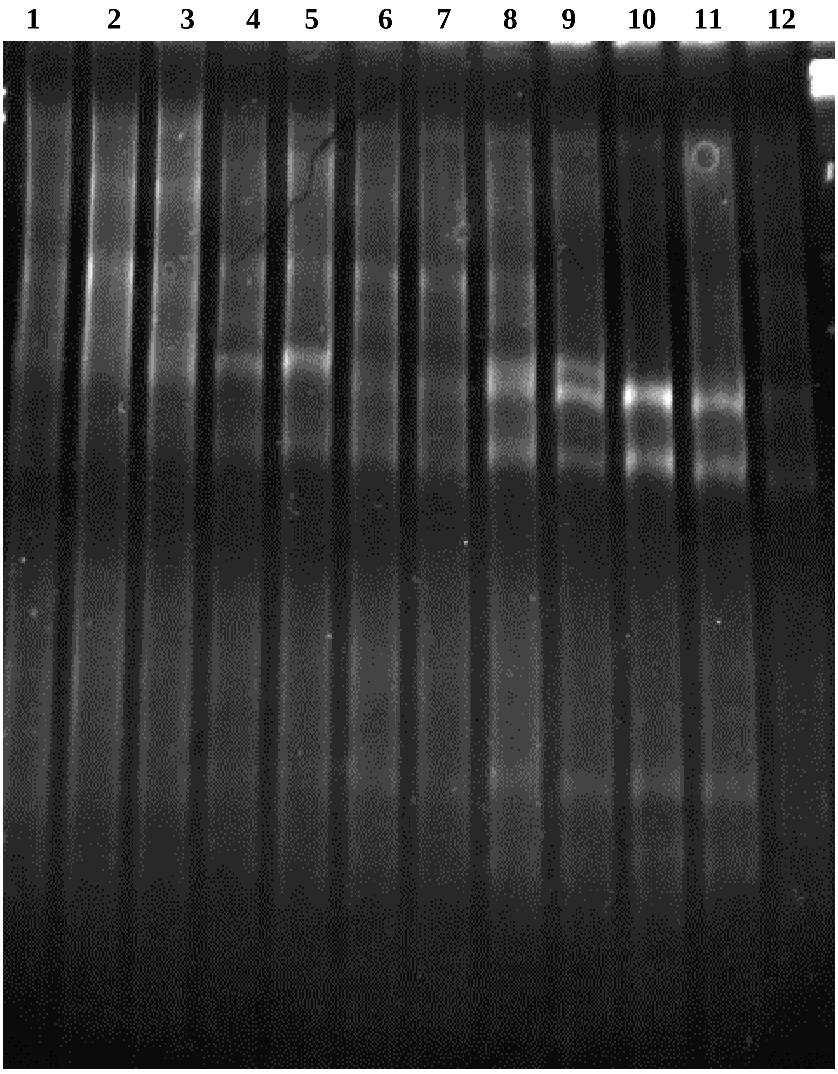


Fig. 1 **DGGE profiles of bacterial community of different enrichment cultures and the mixtures of cyanobacterial strains. Lane 1 – A1 (YMB- T1S 10^{-1}); 2 – A2 (YMB- T2S 10^{-1}); 3 – A3 (YMB- T2S 10^{-6}); 4 - A4 (YMB- T1U 10^{-1}); 5 – A5 (YMB- T1U 10^{-6}); 6 – A6 (YMB- T2U 10^{-1}); 7- A4 (YMB- T2U 10^{-6}); 8 – P1 (Pikovskaya's – T1U 10^{-1}); 9 – P3 (Pikovskaya's – T2U 10^{-6}); 10 – C5 (cyanobacterial mixture of *Anabaena laxa, Calothrix* and *Limnothrix*); 11- C6 (Cyanobacterial mixture of *Anabaena* and *Nostoc* strains (RPAN8, BF1, BF2, BF3 and BF4); 12- C7- (Cyanobacterial mixture of *Anabaena* and *Nostoc* strains - RPAN8, BF1, BF2, BF3 and BF4, along with *Calothrix, Limnothrix*)**

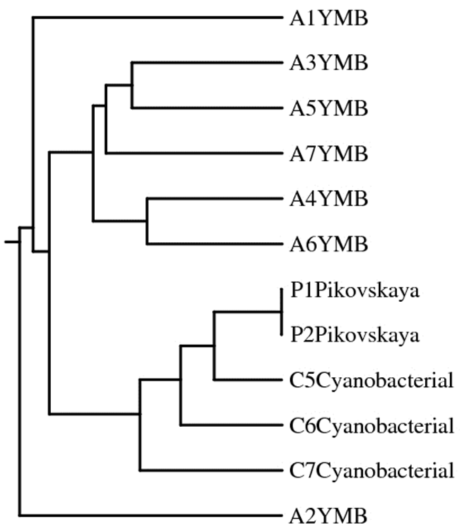


Fig. 2. Dendrogram of bacterial community of different enrichment cultures and the mixtures of cyanobacterial strains. Treatment details: A1 (YMB- T1S 10^{-1}); A2 (YMB- T2S 10^{-1}); A3 (YMB- T2S 10^{-6}); A4 (YMB- T1U 10^{-1}); A5 (YMB- T1U 10^{-6}); A6 (YMB- T2U 10^{-1}); A4 (YMB- T2U 10^{-6}); P1 (Pikovskaya's – T1U 10^{-1}); P3 (Pikovskaya's – T2U 10^{-6}); C5 (Cyanobacterial mixture of *Anabaena laxa, Calothrix* and *Limnothrix*); C6 (Cyanobacterial mixture of *Anabaena* and *Nostoc* strains (RPAN8, BF1, BF2, BF3 and BF4); C7(Cyanobacterial mixture of *Anabaena* and *Nostoc* strains - RPAN8, BF1, BF2, BF3 and BF4, along with *Calothrix, Limnothrix*)

Table 4 Estimation of chlorophyll content and nitrogenase activity in soil samples at 15 DAT

Treatments	**Soil chlorophyll (mg g^{-1} soil)**		**Soil ARA (nmoles ethylene $plant^{-1}$ h^{-1})**	
	Sterile soil	Unsterile soil	Sterile soil	Unsterile soil
Rice plant	2.05 ± 0.069[*]	1.63 ± 0.053	25.42 ± 0.95	28.41 ± 3.60
Rice plant +*Calothrix*	3.19 ± 0.095	1.96 ± 0.035	27.57 ± 0.60	37.63 ± 8.67
SE(m) ±	0.015	0.038	0.432	2.450
CD($P \leq 0.05$)	0.040	0.110	1.200	6.790

[*] All values represent means ± SD; n=3

tissues of the *Calothrix* inoculated plants grouped together. The bacterial communities of control plants' root was distinctly different from that of *Calothrix* inoculated plant roots (Fig. 3).

The DGGE patterns representing bacterial communities of root and leaf tissues of rice and bulk soils under sterile or unsterile conditions were given in (Fig 4). In order to understand the similarity pattern of bacterial communities in leaf- and root tissues or soils separately, the cluster analyses were performed by considering only the banding pattern of individual lanes. The bacterial communities of leaf tissues of plants on 15 DAT and with cyanobacterial inoculation showed similar patterns; the communities of leaf tissues of plants inoculated with cyanobacterium, under either sterile or unsterile conditions were similar. While the community patterns of leaf tissues of plants without inoculation on 15 and 45 DAT could be grouped together, the patterns of leaves from unsterile- and sterile soils on 15 and 45 DAT showed similarity. When the community patterns of bulk and rhizosphere soils were analyzed, the patterns of sterile bulk and rhizosphere soils on 45 DAT were found to be similar. Interestingly, the sterile bulk soil on 15 DAT and the rhizosphere soil after cyanobacterial inoculation on 45 DAT showed similarity, evident from the distinct clusters (Fig 5). Profiles corresponding to sterile and unsterile conditions due to cyanobacterial inoculation could not be grouped together, probably because of the differences in intensities and subsequently on the detection of bands in the individual lanes.

Characterization of microbial communities by PLFA analysis

The concentrations of total PLFAs ranged from 45 to 170 nmoles g^{-1} in soil after 15 d of transplanting (DAT) with/without cyanobacterial inoculation. The bulk soil under unsterile condition had a higher concentration of total PLFAs than that of sterile condition (Fig.6). In general, the rhizosphere soils of both sterile and unsterile conditions had higher amounts of total PLFAs compared to the respective bulk soils. The rhizosphere soil had higher amounts of total PLFAs due to cyanobacterial inoculation, more so under the unsterile condition Likewise, the cyanobacterial inoculation increased the total concentration of PLFAs by about two fold in the bulk soils. Total concentrations of PLFAs increased by 3 to 16 folds on 45 DAT, compared

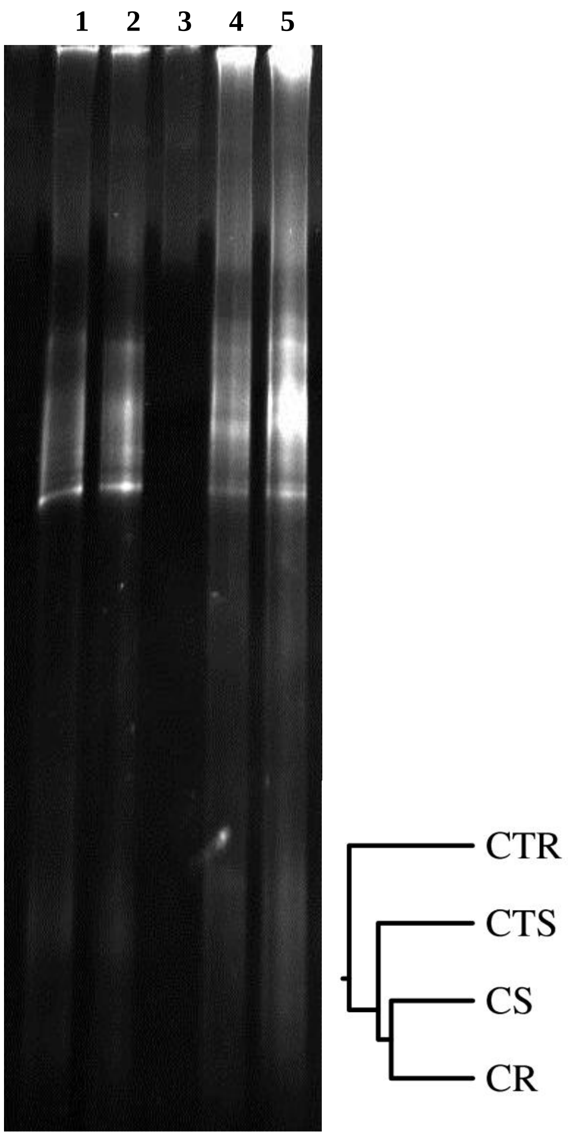


Fig. 3 **DGGE profile and dendrogram of bacterial community of rice plant with or without *Calothrix elenkinii* inoculation, in the water agar microcosm experiment. Lane 1 CTR – Control root; 2 CTS- Control shoot; 3- Blank; 4- CS- *Calothrix* inoculated rice shoot; 5- CR- *Calothrix* inoculated rice root**

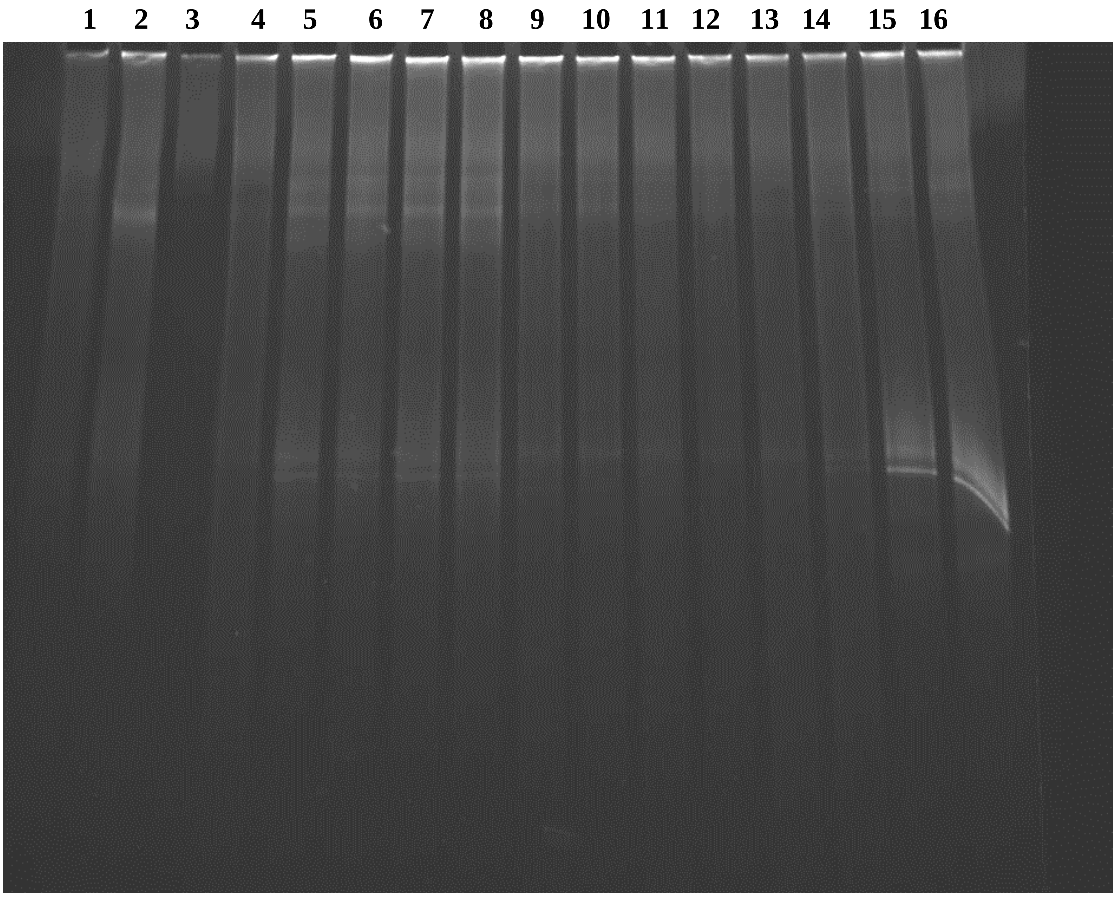
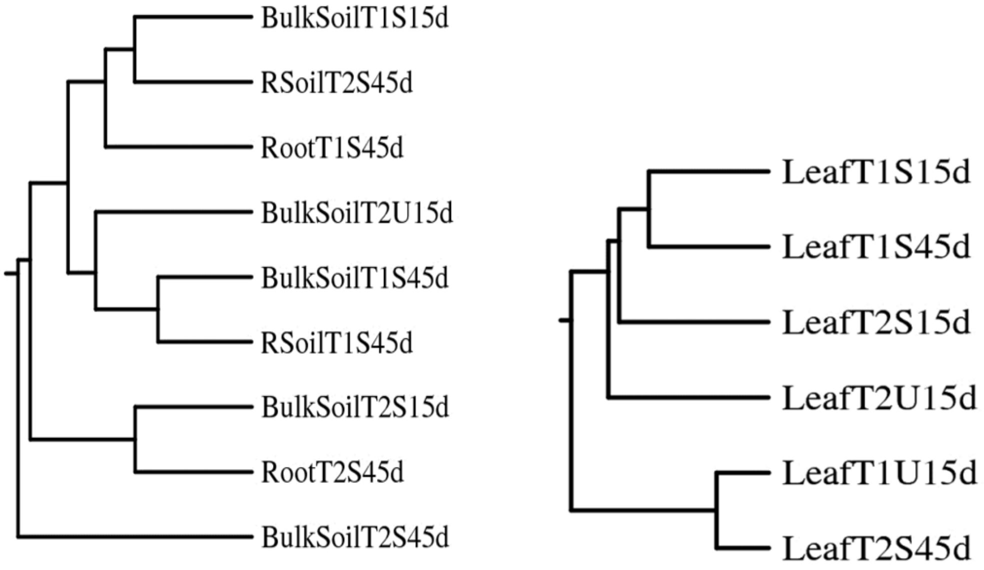


Fig. 4 DGGE profiles of bacterial community of leaf and root microbiome of rice with or without cyanobacterial inoculation

Lane 1 – Bulk Sterile soil T1 (- *Calothrix*) 15DAT; 2- Bulk Sterile soil T2 (+ *Calothrix*); 15 DAT; 3- Blank; 4- Bulk Unsterile soil T2 (+*Calothrix*) 15 DAT; 5- Leaf T1(Sterile - *Calothrix*) 15DAT; 6- Leaf T2 (Sterile+ *Calothrix*) 15DAT; 7 - Leaf T1(Unsterile - *Calothrix*) 15DAT; 8- Leaf T2 (Unsterile+ *Calothrix*) 15DAT; 9 - Bulk Sterile soil T1 (- *Calothrix*) 45DAT; 10- Bulk Sterile soil T2 (+ *Calothrix*) 45 DAT; 11- Rhizosphere Sterile soil T1 (- *Calothrix*) 15DAT; 12- Rhizosphere Sterile soil T2 (+ *Calothrix*); 13- Root T1 (Sterile, - *Calothrix*) soil 15 DAT; 14- Root (Sterile, + *Calothrix*) 15 DAT; 15- Leaf T1 (Sterile - *Calothrix*) 45 DAT; 16- Leaf T2 (Sterile, + *Calothrix*) 45 DAT



A B

Fig. 5 Dendrogram of bacterial community of soil/root- and leaf microbiome of rice with or without inoculation. d refers to DAT (days after transplanting). Details of labels of clusters:

A: Soil/root microbiome

BulkSoilT1S-15 DAT d: Bulk Sterile soil T1 (- *Calothrix*) 15 DAT; BulkSoilT2S-15 DAT- Bulk Sterile soil T2 (+ *Calothrix*) 15 DAT; BulkSoilT2U-15 DAT-Bulk Unsterile soil T2 (+*Calothrix*) 15 DAT; BulkSoilT1S- 45 DAT- Bulk Sterile soil T1 (- *Calothrix*) 45 DAT; BulkSoilT2S-45 DAT- Bulk Sterile soil T2 (+ *Calothrix*) 45 DAT; RhizosphereSoilT1S-15 DAT- Rhizosphere Sterile soil T1 (- *Calothrix*) 15 DAT; RSoilT2S-45DAT- Rhizosphere Sterile soil T2 (+ *Calothrix*); RootT1S-15 DAT - Root T1 (Sterile, - *Calothrix*) soil 15 DAT; RootT2S-45 DAT - Root (Sterile, + *Calothrix*) 15 DAT

B: Leaf microbiome

LeafT1S-15 DAT- Leaf T1 (Sterile - *Calothrix*) 15 DAT; LeafT2S-15 DAT - Leaf T2 (Sterile+ *Calothrix*) 15 DAT; LeafT1U- 15 DAT - Leaf T1(Unsterile - *Calothrix*) 15DAT; LeafT2U-15 DAT - Leaf T2 (Unsterile + *Calothrix*) 15 DAT; LeafT1S-45 DAT - Leaf T1 (Sterile - *Calothrix*) 45 DAT; LeafT2S-45 DAT - Leaf T2 (Sterile, + *Calothrix*) 45 DAT

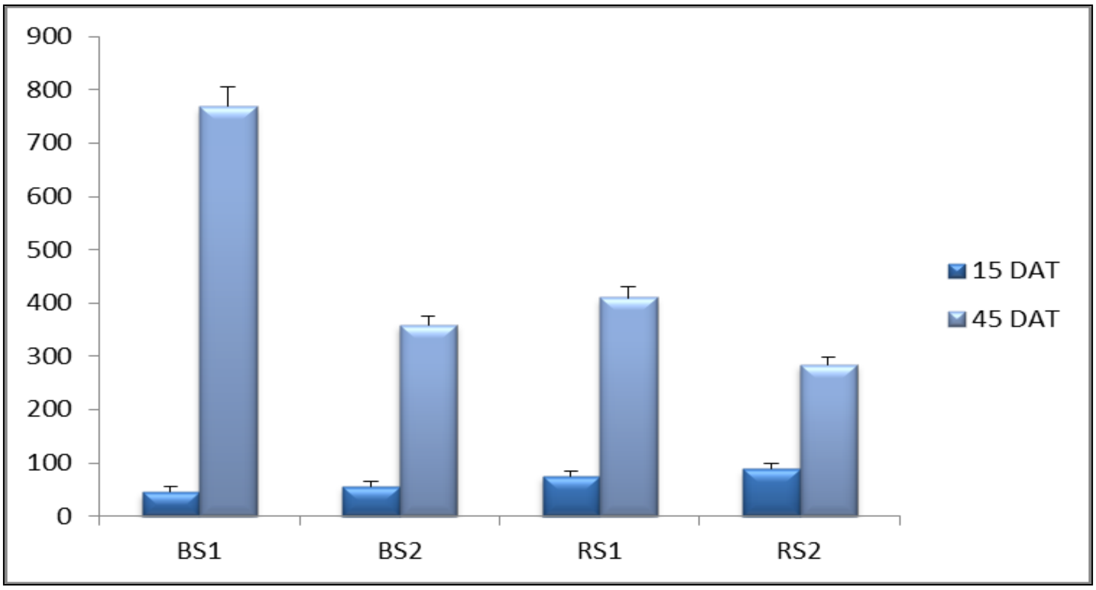
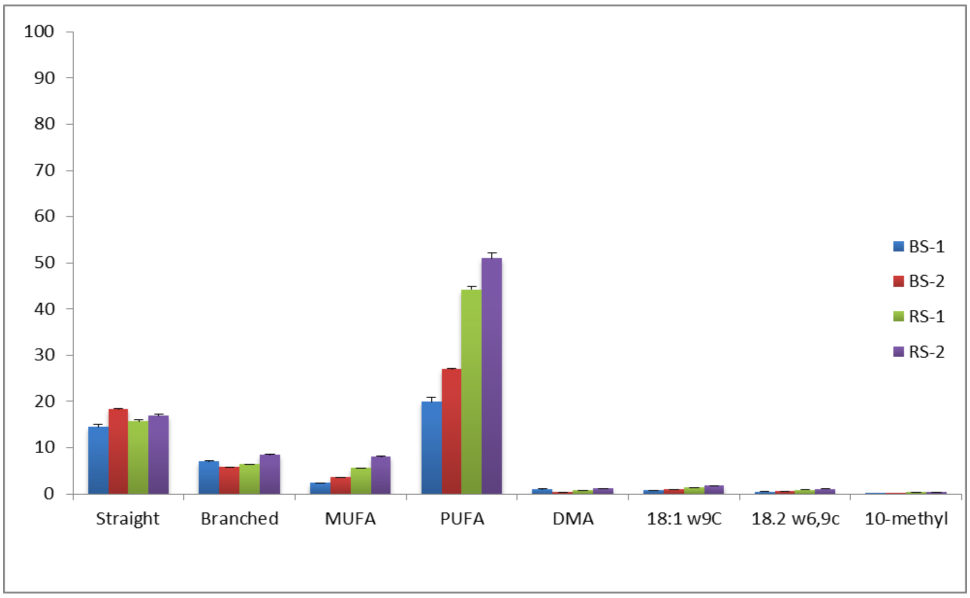


Fig. 6 Changes in microbial biomass as represented by total phospholipid fatty acids (PLFAs in nmoles g^{-1}) on 15 and 45 DAT of cyanobacterial inoculation

to that of soils on 15 DAT. Highest amount of total PLFAs (768 nmoles g^{-1}) was observed in the bulk soil without cyanobacterial inoculation under sterile condition. In contrast to the effect of cyanobacterial inoculation on 15 DAT, there were decreases in the total PLFAs compared to the respective control treatments on 45 DAT.

On 15 DAT, the concentrations of total PLFAs were more in unsterile than sterile soils, with or without cyanobacterial inoculation. The fatty acid types in unsterile soils were characteristically different, illustrating the influence of cyanobacterial inoculation (Fig 7). Polyunsaturated- and straight chain fatty acids in unsterile soils were relatively more than that of sterile soils. In the bulk soil under the unsterile condition, the mixed- (18:1 ω9c and 18:2 ω6.9c), monounsaturated and straight fatty acids increased, but the branched and polyunsaturated fatty acids decreased due to cyanobacterial inoculation. On the contrary, there were decreases in the concentrations of 18:1 ω9c, 18:2 ω6.9c, 10-methyl-, dimethyl acetal and polyunsaturated fatty acids and, increases in straight, branched and monounsaturated fatty acids (MUFA) in the cyanobacteria-inoculated rice rhizosphere under unsterile condition (Fig 8). But, the relative changes in different fatty acid types were smaller in the sterile bulk- and rhizosphere soils due to the cyanobacterial inoculation. When the analysis was performed on the basis of biomarker PLFAs, the absolute concentrations in different microbial guilds suggested the positive influence of cyanobacterial inoculation, more under unsterile than under sterile soil conditions (Fig 9). But the relative changes in the microbial communities distinctly differed among the sterile and unsterile bulk and rhizosphere soils due to cyanobacterial inoculation (Fig 10). These changes were also evident from the cluster analysis of PLFA profiles because the profiles of rhizosphere with or without cyanobacterial inoculation grouped together. The cyanobacterial inoculation brought about distinct differences in the PLFA profiles between the sterile and unsterile bulk soils. Principal Component Analysis was applied to classify these PLFA profiles based on the overall pattern of correlation within the fatty acid types and soil under various treatments (Fig. 11). The first two principal components accounted for 79% of the total variance among the types. However, the score plot and the loading

A



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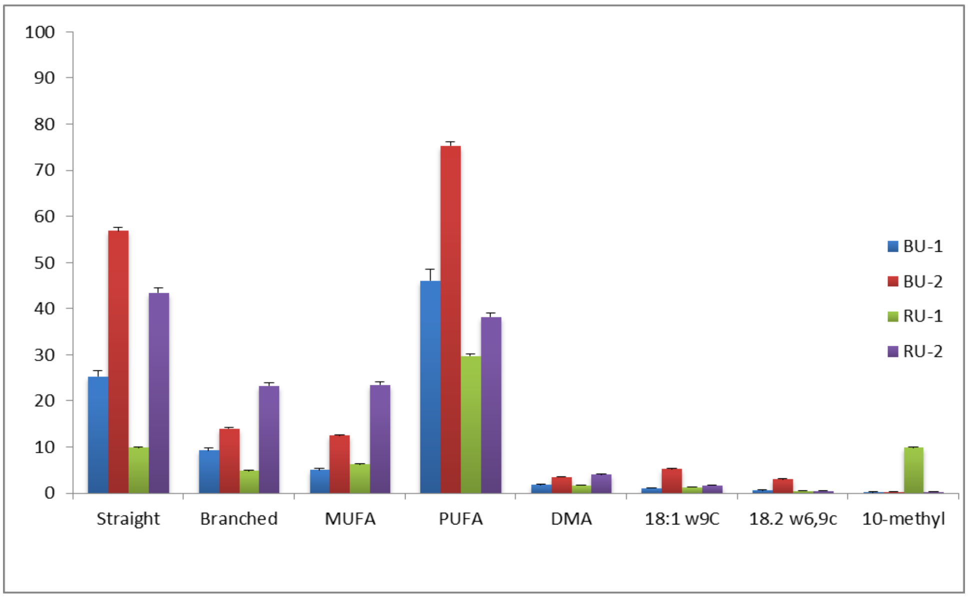


Fig. 7 Abundance of different fatty acid types (nmoles g^{-1}) in Sterile (A) and Unsterile soil (B) planted to rice with or without cyanobacterial inoculation on 15 DAT

[BS-1: Sterile Bulk Soil without cyanobacterial inoculation; BS-2: Sterile Bulk Soil with cyanobacterial inoculation; RS-1: Sterile Rhizosphere soil without cyanobacterial inoculation; RS-2: Sterile Rhizosphere soil with cyanobacterial inoculation; BU-1: Unsterile Bulk Soil without cyanobacterial inoculation; BU-2: Unsterile Bulk Soil with cyanobacterial inoculation; RU-1: Unsterile Rhizosphere soil without cyanobacterial inoculation; RU-2: Unsterile Rhizosphere soil with cyanobacterial inoculation]

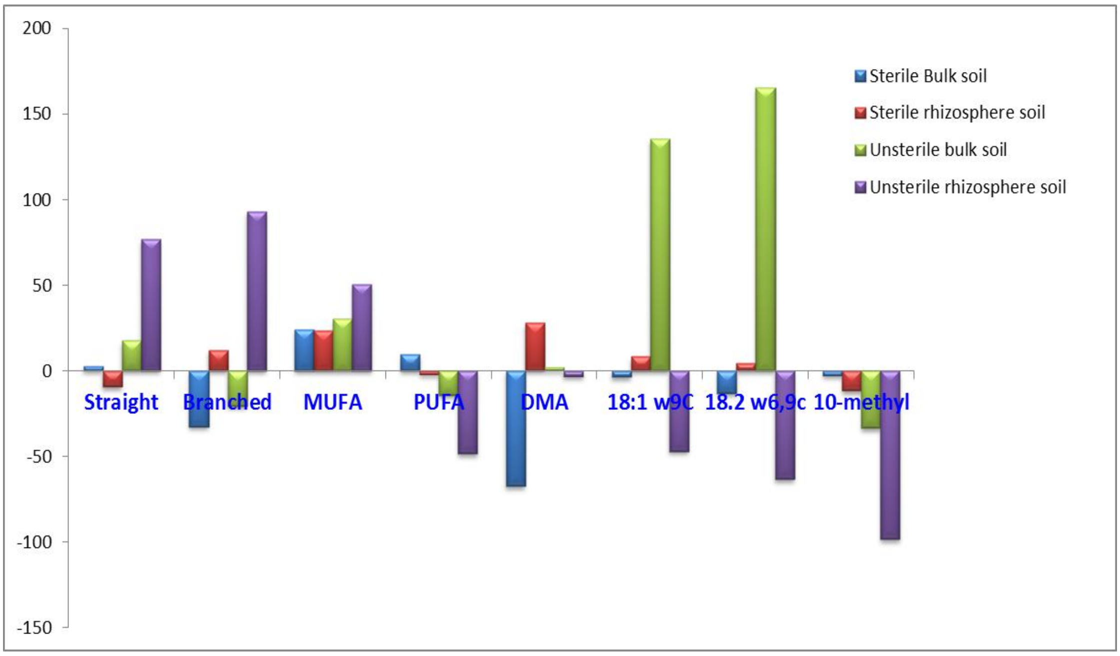
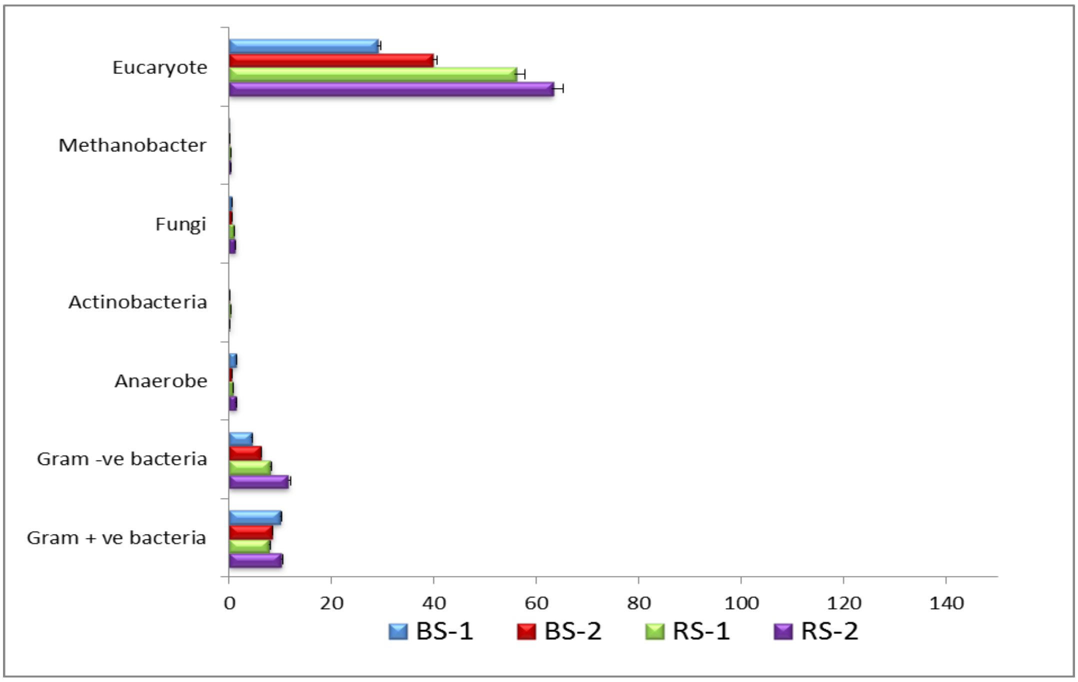


Fig. 8 Relative changes (%) in different fatty acid types (nmoles g^{-1}) in Sterile and Unsterile soil planted to rice due to cyanobacterial inoculation on 15 DAT

A



B

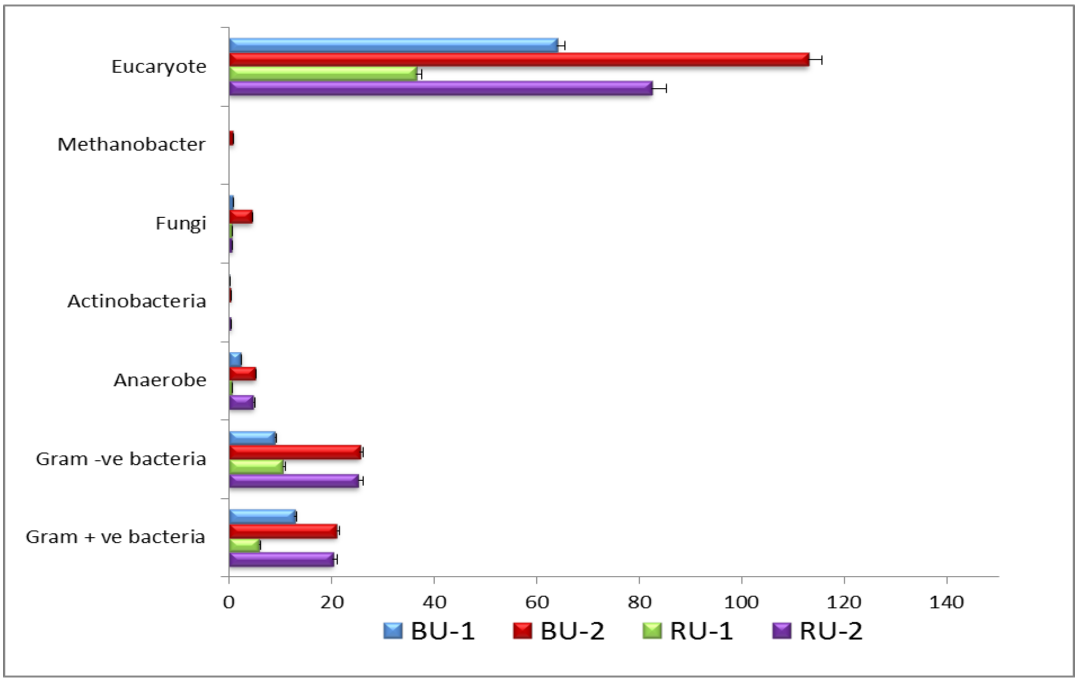


Fig. 9 Abundance of different microbial 'biomarker' phospholipid fatty acids (nmoles g^{-1}) in Sterile (A) and Unsterile soil (B) planted to rice with or without cyanobacterial inoculation on 15 DAT.

[BS-1: Sterile Bulk Soil without cyanobacterial inoculation; BS-2: Sterile Bulk Soil with cyanobacterial inoculation; RS-1: Sterile Rhizosphere soil without cyanobacterial inoculation; RS-2: Sterile Rhizosphere soil with cyanobacterial inoculation; BU-1: Unsterile Bulk Soil without cyanobacterial inoculation; BU-2: Unsterile Bulk Soil with cyanobacterial inoculation; RU-1: Unsterile Rhizosphere soil without cyanobacterial inoculation; RU-2: Unsterile Rhizosphere soil with cyanobacterial inoculation]

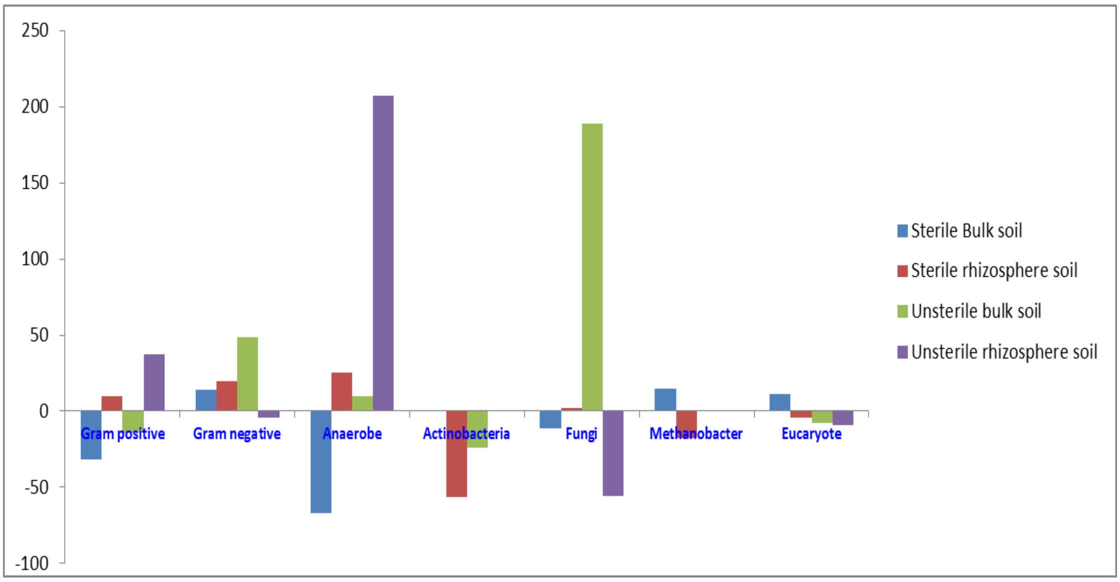
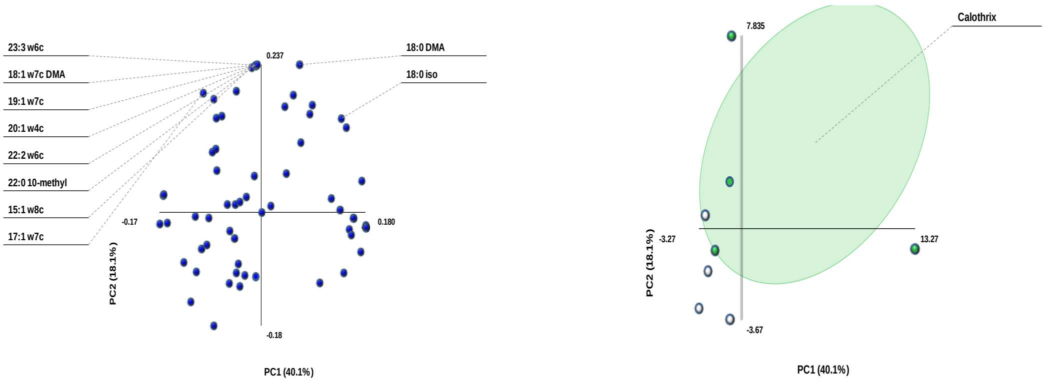
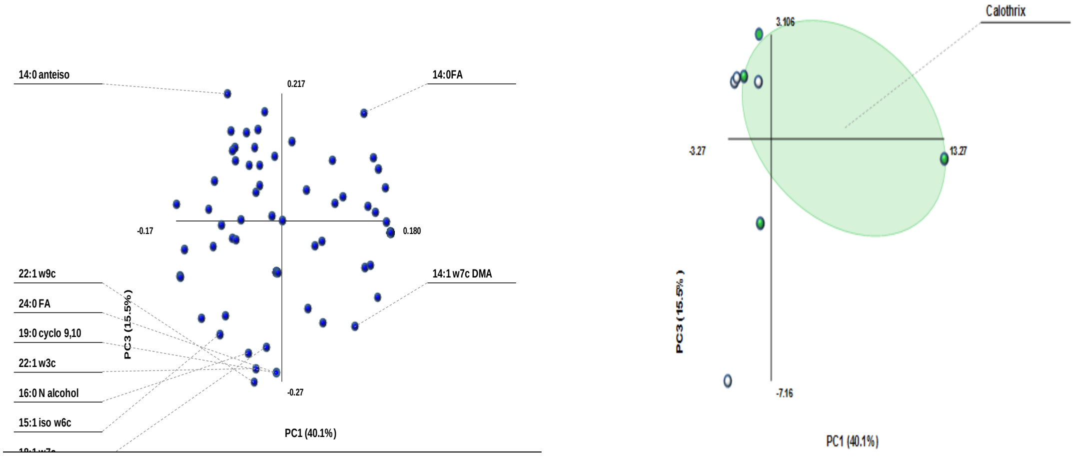


Fig. 10 Relative changes (%) in different microbial biomarker phospholipid fatty acids (PLFAs) (nmoles g^{-1}) in Sterile and Unsterile soil planted to rice due to cyanobacterial inoculation on 15 DAT

PCA1 vs PCA2



PCA1 vs PCA3



PCA2 vs PCA3

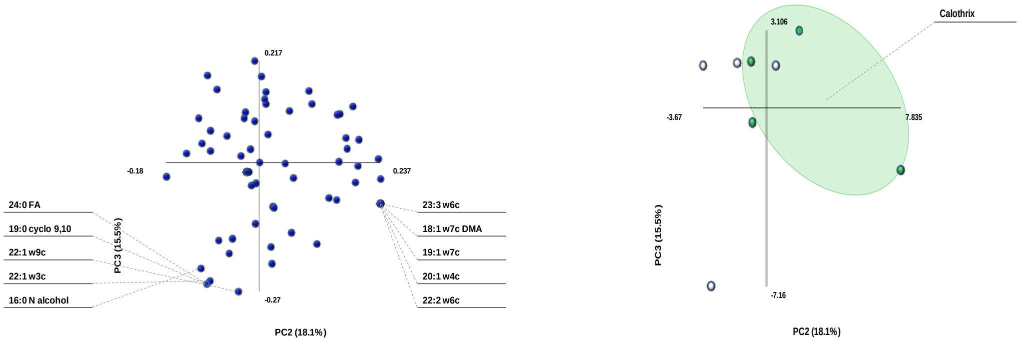


Fig. 11 Principal component analysis of different PLFA profiles of soils planted to rice with or without cyanobacterial inoculation on 15 DAT.

plot suggested that there were no clear differences in PLFAs due to cyanobacterial inoculation on 15 DAT (Fig.12).

The concentrations of total PLFAs in sterile bulk or rhizosphere soils ranged between 280 and 768 nmoles g^{-1} on 45 DAT (Fig.6). Polyunsaturated fatty acids comprised of 30-40%, followed by straight chain fatty acids (20-29%) and monounsaturated fatty acids (7-18%). The absolute concentrations of different fatty acids showed decreases due to cyanobacterial inoculation (Fig.13). Interestingly, the relative proportions of fatty acid types such as straight, monounsaturated, and polyunsaturated (PUFA) showed increases due to cyanobacterial inoculation (data not shown). However, the absolute concentrations of ‘biomarker PLFAs’ also showed decreases due to cyanobacterial inoculation. The decreases in the absolute concentrations of biomarker PLFAs belonging to eukaryotes were more in bulk soil than in the rhizosphere soil (Fig.14). There were also decreases in the relative abundance of all other biomarker PLFAs including that of Gram-negative bacteria (67-70%), anaerobe (33-57%) and Gram-positive bacteria (11-49%) due to cyanobacterial inoculation (Fig. 15). The PCA analysis of the PLFA profiles clearly separated the cyanobacterial inoculation in sterile soils on 45 DAT. The first two principal components accounted for 75% of the total variance. The score plots of PCA1 versus PCA2, and PCA2 versus PCA3 suggested the influences of cyanobacterial inoculation on the concentration of different fatty acid types (Fig. 16).

Discussion

Cyanobacteria represent a commonly used input in rice cultivation, due to their frequent presence in rice paddies, exhibiting not only a wide geographic and regional distribution, but also tremendous taxonomic and molecular diversity (Mandal *et al.* 1998; Irisarri *et al.* 2001; Prasanna *et al.* 2012a,b, 2013a, 2014b). The rice plant represents a habitat for diverse micro-organisms, colonizing the zone around the root (rhizosphere) and the root surface (rhizoplane), besides the aerial parts (phyllosphere) (Knief *et al.* 2012). The diversity and activity of microbial communities in the rhizosphere of irrigated rice soils influence the soil fertility and nutrient use efficiency. However, the influence of microbial inoculation on the microbiome of rice is a less

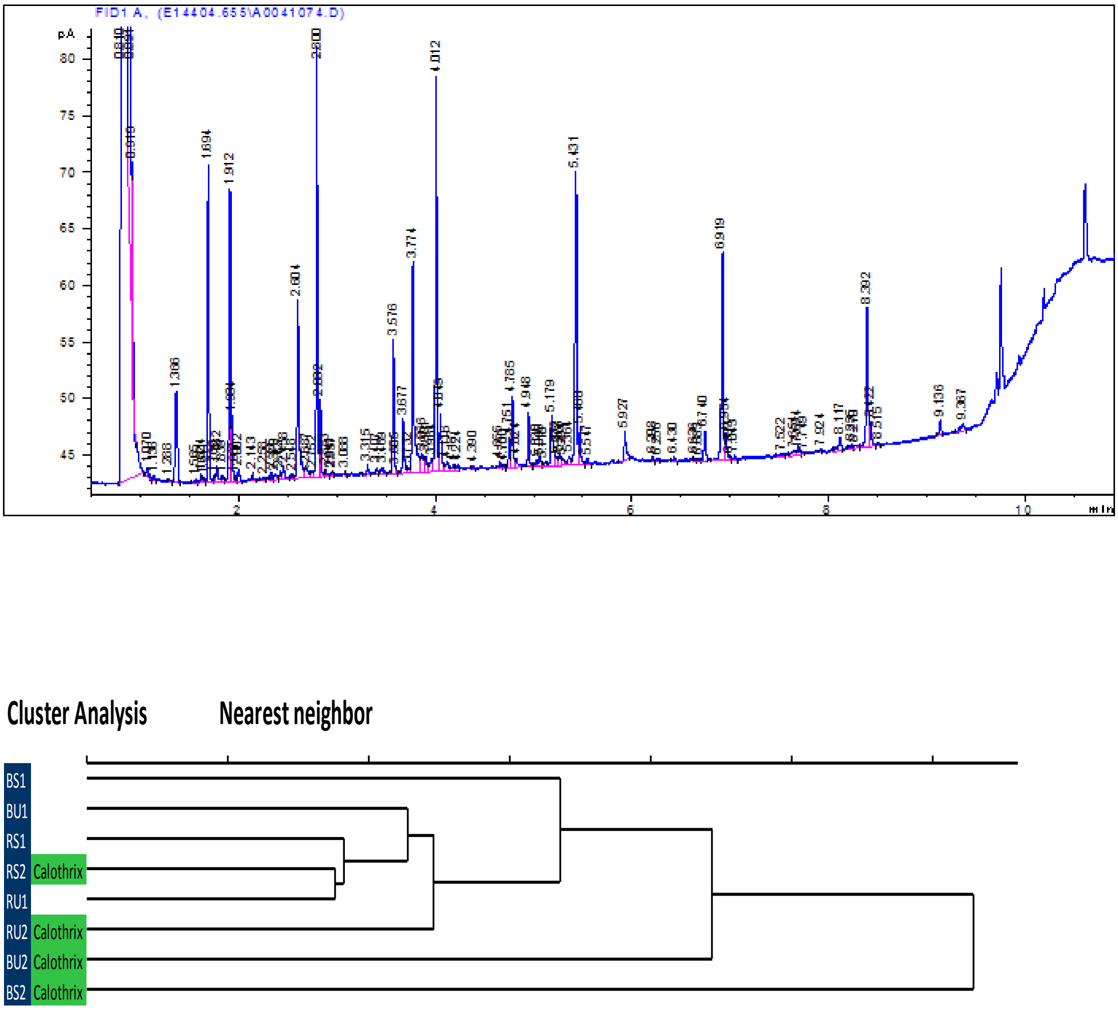


Fig.12 Representative chromatogram used for multivariate statistical analysis and clustering of different PLFA profiles obtained on 15 DAT.

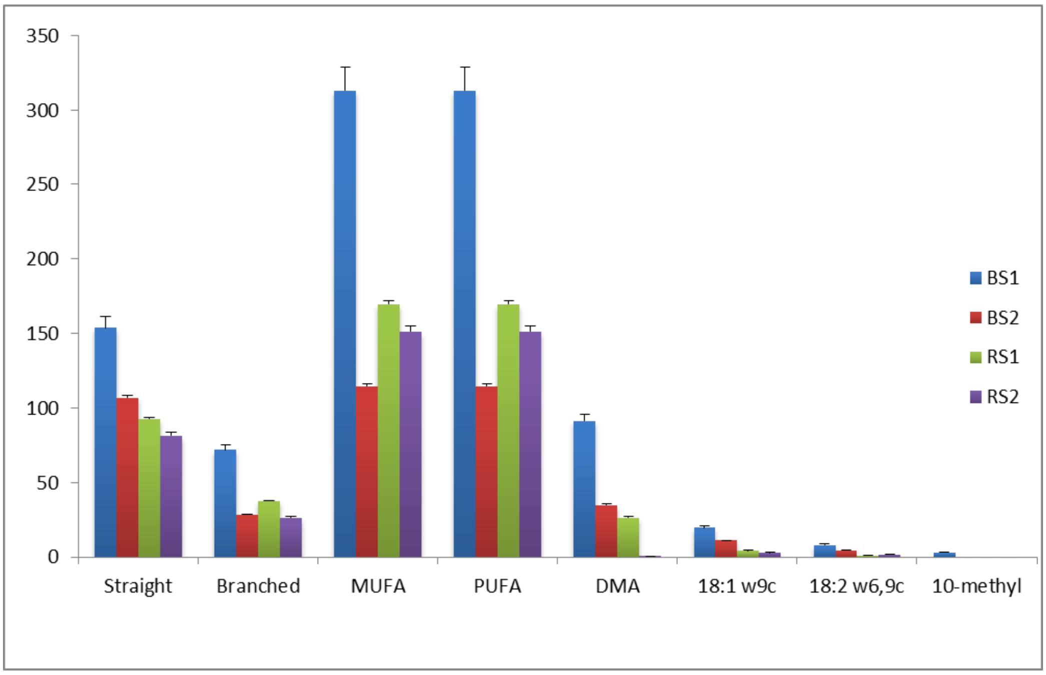


Fig.13 Abundance of different fatty acid types (nmoles g^{-1}) in Sterile soil planted to rice with or without cyanobacterial inoculation on 45 DAT

[BS1: Sterile Bulk Soil without cyanobacterial inoculation; BS2: Sterile Bulk Soil with cyanobacterial inoculation; RS1: Sterile Rhizosphere soil without cyanobacterial inoculation; RS2: Sterile Rhizosphere soil with cyanobacterial inoculation]

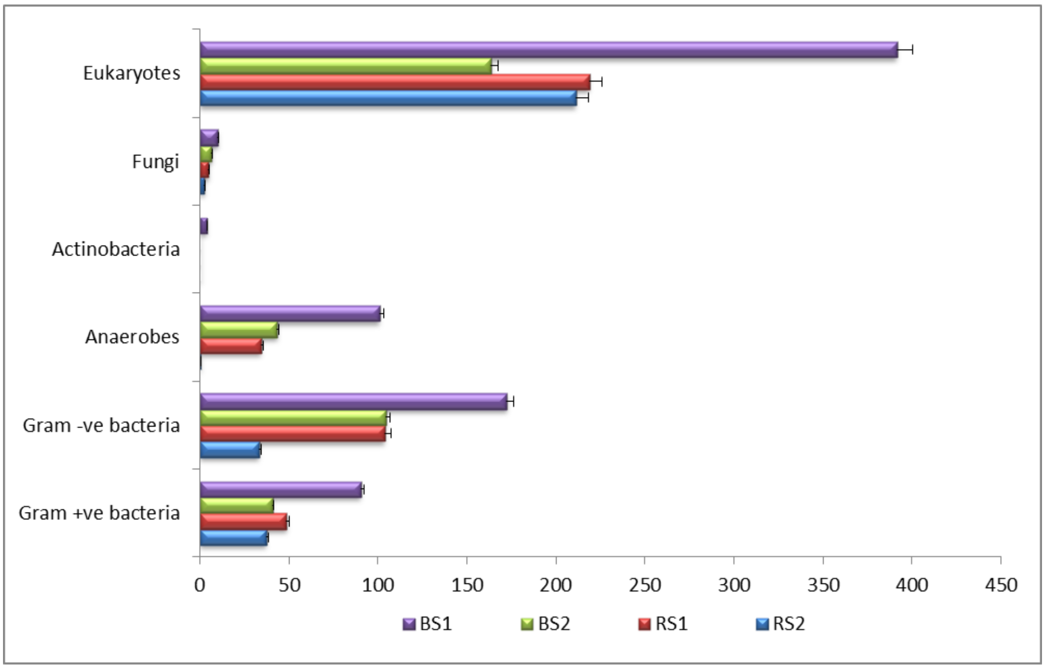


Fig.14 Abundance of different microbial 'biomarker' phospholipid fatty acids (nmoles g^{-1}) in Sterile planted to rice with or without cyanobacterial inoculation on 45 DAT.

[BS1: Sterile Bulk Soil without cyanobacterial inoculation; BS2: Sterile Bulk Soil with cyanobacterial inoculation; RS1: Sterile Rhizosphere soil without cyanobacterial inoculation; RS2: Sterile Rhizosphere soil with cyanobacterial inoculation]

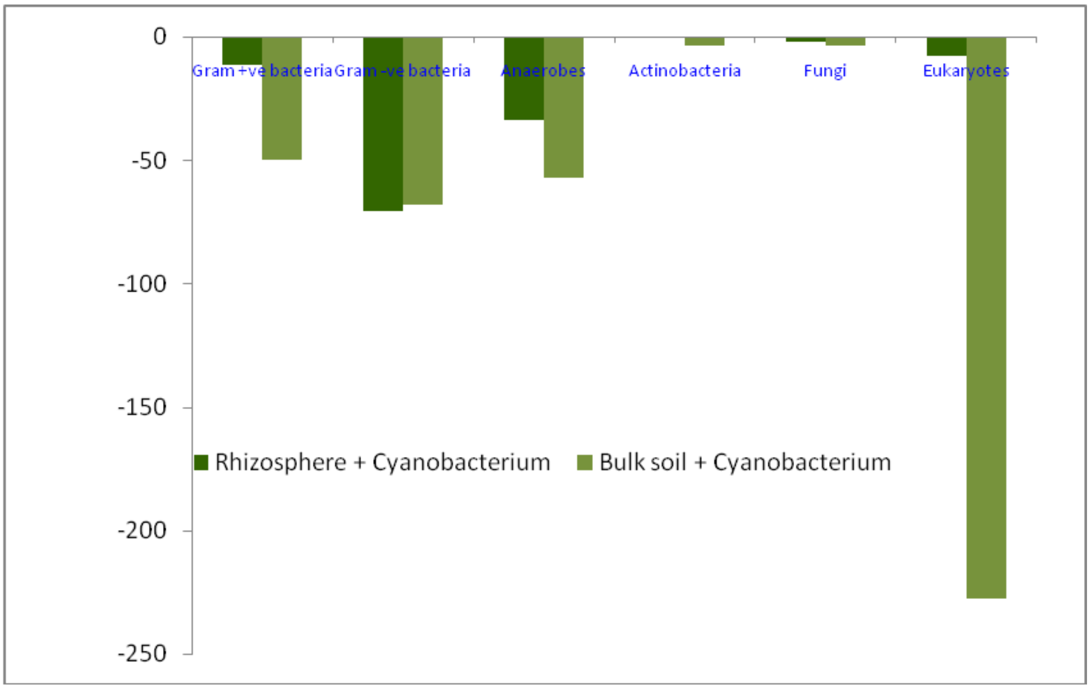
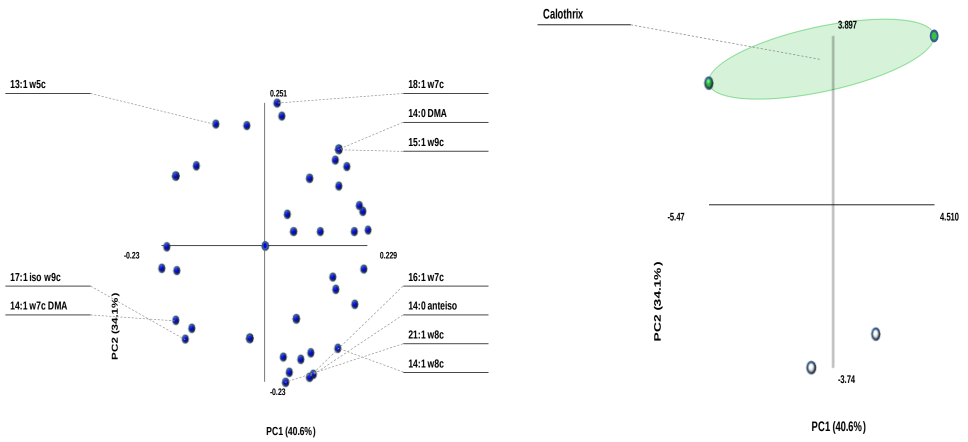
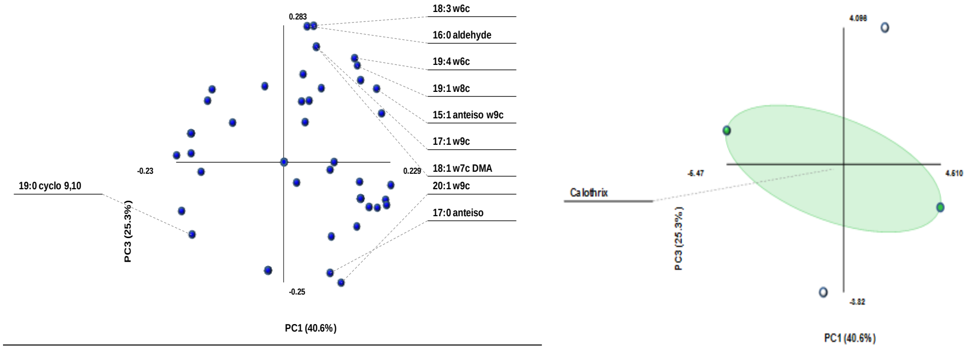


Fig. 15 Relative changes (%) in different microbial biomarker phospholipid fatty acids (PLFAs) (nmoles g^{-1}) in Sterile soil planted to rice by cyanobacterial inoculation on 45 DAT

PCA 1 vs PCA2



PCA1 vs PCA3



PCA2 vs PCA3

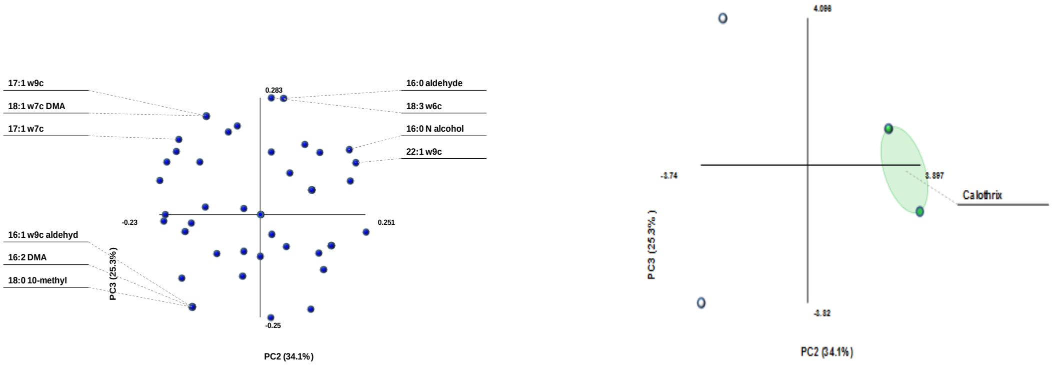


Fig.16 Principal component analysis of different PLFA profiles of soils planted to rice with or without cyanobacterial inoculation on 45 DAT.

investigated area. Cyanobacterial biofertilizers can be important inputs for increasing crop productivity to the extent of 10-24% in diverse locations in the world, especially South East Asia; this has been attributed to a variety of other factors, besides nitrogen fixation, improving plant growth by producing IAA, improving soil structure through the secretion of extracellular polysaccharides and increasing the carbon and nitrogen status of soil (De Caire *et al*. 2000; Prasanna *et al.* 2012a; Mandal *et al*. 1998; Irisarri *et al.* 2001; Prasanna *et al.* 2012a, b; Prasanna *et al*. 2013b, 2014 a,b). They produce plant growth promoting substances and assimilate organic compounds (Sergeeva *et al*., 2002), but their associations with plants, more specifically the microbiome of crop plants has not been investigated in relation to their utilization in agriculture.

Therefore, an investigation was undertaken to monitor the effect of cyanobacterial inoculation on the soil and plant microbiome of rice, employing microbiological and culture-independent molecular tools in pot experiments, using sterile and unsterile soil. Analyses of biometrical parameters 15 and 45 DAT revealed 20-30% enhancement in plant length, fresh and dry weight of plants. These observations were in accordance with the levels of available N and P in soil which showed a similar trend in sterile and unsterile soil. The soil samples also recorded higher organic C (%) at 45 DAT. The nitrogen fixing potential, measured as ARA at 15 DAT showed a significant increase in inoculated soil, particularly unsterile soil. Soil chlorophyll, which represents an index of photosynthetic biomass accretion, showed a 20-50% increase in *Calothrix* sp. inoculated soil. Jaiswal *et al.*, (2008) had recorded a similar enhancement in soil and plant chlorophyll in co-inoculation experiments with wheat seedlings, as the cyanobacterial strains produce IAA. This is similar to our observations and as recorded earlier in cyanobionts, symbiotic, free-living cyanobacteria (Stewart *et al.,* 1983; Sergeeva *et al.,* 2002). Cyanobacterial inoculation is known to enhance plant growth and improve nutrient mobilization in soil and its uptake by plants (Prasanna *et al.* 2012a,b, 2014a,b). A significant enhancement in microbial activity, and plant growth/yields and savings of 25% N in wheat-rice cropping sequence was recorded with the use of cyanobacterial formulations (Prasanna *et al.* 2012c). Such formulations significantly enhanced organic carbon, nitrogen fixation in soil and its uptake by rice and wheat plants. Hitherto, it is not known whether the changes due to a single cyanobacterial inoculation caused the response of microbial communities by altering the diversity or abundance of the constituent members in rhizosphere or bulk soils.

The rice soil ecosystems are heterogeneous and highly complex, not only with their physic-chemical conditions but also their microbial communities. The gradients of oxygen and other electron acceptors make these ecosystems highly favourable for the growth of facultative and anaerobic microorganisms. Hence the conventional enumeration procedures for microorganisms under aerobic conditions are inadequate to account all the members of microbial communities. Earlier reports suggest that total prokaryotic abundance which includes bacteria and archaea can range between 4 and 7 × 10^9 cells g^{-1} in the dry paddy soil, with about 44% accounting for aerobic, and 32% for facultative anaerobic bacteria and 24% for archaea (Bai *et al.* 2000). Since only about 1% of the soil bacterial population can be cultured (Torsvik *et al.* 1998), it is difficult to assume that the culturable microorganisms represent all the members of microbial communities adequately. Even among those bacteria which are observed under a microscope, about 99% of them could not be cultured by the common laboratory techniques (Trevors *et al.* 1998; Kirk *et al.* 2004). In a study on the spatial heterogeneity of soil microbial communities in agricultural soil, Franklin and Mills (2003) showed that there existed several nested levels of organization depending on soil properties or group of properties. The spatial distribution of bacteria is also influenced by the presence of plants, with about two-fold increase in rhizosphere over bulk soil (Curl and Truelove 1986). Besides, there exists a high level of phenotypic and genetic diversity among bacterial and fungal communities in soils. The development of methods for 16S rRNA sequence analysis improved the detection and identification of many bacteria in nature and confirmed that only a small percentage of bacterial species has been isolated in culture. PCR targeting the 16S rRNA sequences have led to many methods of molecular fingerprinting of bacterial communities including DGGE patterns.

The DGGE analysis of 16S rRNA gene segments has been widely accepted to profile the complex microbial communities (Muyzer *et al.* 1993; Inceoglu *et al.* 2013).

The results from the present study showed that the DGGE profiles of enrichment cultures and the mixtures of cyanobacteria can also be used to study the known populations. The bacterial community patterns of enrichments using the Yeast Extract Mannitol broth were found to be similar except for that of the community of the A2 (YMB). The similar community patterns between those of Pikovskaya enrichments and the mixtures of cyanobacteria could not be discerned. These difficulties could be due to the differences in the number of rRNA operons within a single- or multiple organism populations. The major advantage of DGGE profiling of bacterial communities is the detection of phylogenetically related populations as a consistent pattern. Such patterns of phylogenetically related populations support the generality of the ecological basis for bacterial diversity (Ferris et al. 1996). In the present study, the DGGE profiles corresponding to sterile and unsterile conditions due to cyanobacterial inoculation could not be always grouped together. This difficulty could be probably due to the true reflection of the populations at the sampling time or the bias introduced in the PCR amplification of certain sequence types. Besides, the analysis of microbial communities using soil samples of 1 to 5 g may favor the detection of only the dominant population. Nevertheless, the DGGE profiling provides a rapid means of screening large numbers of samples for the spatiotemporal distributions of bacterial communities. Recently, Murase *et al.* (2014) applied the DGGE method targeting the 18S rRNA gene or reverse-transcribed 18S rRNA and showed that the oxygen availability was an important determinant of microeukaryotic community, shifts observed within a week of incubation of a Japanese rice field soil.

The PLFA profiles of soil organisms helped to study the microbial community structure by avoiding the limitations due to primer designs for different microbial groups and PCR amplification itself. The microbial biomass measurements using the phospholipid approach showed that there were declines due to cyanobacterial inoculation on 45 DAT. Earlier report of Reichardt *et al.* (1997) showed that total microbial biomass expressed as total PLFAs decreased at the flowering stage of rice. The rice plants may have intrinsic capabilities to regulate the microbial abundance during the flowering stage in order to minimize the transfer of photosynthate inputs to rhizosphere (Lu *et al.* 2004). The present study showed that the cyanobacterial inoculation and subsequent colonization of rice roots probably favoured the maintenance of only the 'core microbiome.' The individual fatty acid types of different PLFA profiles derived from soils on 15 DAT and 45 DAT clearly showed the alterations within the members of the major domains such as Bacteria and Eukarya. These PLFA profiles confirmed that the cyanobacterial inoculation led to decreases in the constituent members such as Gram-negative bacteria (67-70%), anaerobe (33-57%) and Gram-positive bacteria (11-49%). Not all the microbial guilds of identified by the PLFA markers responded to the cyanobacterial inoculation consistently in both the bulk and rhizosphere soils, probably due to the inherent changes in the microbial community structure in the beginning. The multivariate statistical analysis such as the principal component analysis (PCA) of the whole PLFA pattern also supported the influence of cyanobacterial inoculation on 45 DAT. Earlier, Bossio and Scow (1998) found a decrease in fungal and aerobic bacterial indicators, with an increase in Gram-positive bacterial PLFA markers due to flooding. Unger *et al.* (2009) also demonstrated that flooding decreased microbial biomass and the PLFA markers for aerobic bacteria, Gram-negative bacteria, Gram-positive bacteria, and mycorrhizal fungi. In another study, Zhao *et al.* (2014) showed by 16S rRNA gene amplification and 454 pyrosequencing that the fertilizer application had a significant effect on the variation and composition of bacterial community in the rice-wheat cropping system. The decreased presence of Gram-negative bacteria, anaerobe and Gram-positive bacteria due to cyanobacterial inoculation in the current study provides new evidence on the changes in the bacterial community structure.

Our study clearly demonstrated that the cyanobacterial inoculation affected the soil nutrient cycling, activities and the community structure of microorganisms and plant growth favorably. There are declines in the abundance of major members within the community structure of rhizosphere due to cyanobacteria-inoculation, which was actually beneficial to the plant growth. Further study is warranted in identifying the members of 'core microbiome,' which are an important determinant of the growth and development of rice.

Acknowledgements

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6. DISCUSSION

Rice is the primary cereal crop which constitutes the major nutrient source for more than two-fifth of world's population. As a major crop of the Asia-Pacific Region, it provides food security to the growing human populations. Rice has been cultivated for thousands of years in Asia. More than 50% of the area under rice comprises intensive irrigated systems that account for 75% of its global production, and represent the home of the Green Revolution. As rice continues to supply 50-80 percent of the daily calories, the average growth rate in production has to keep pace with the human population growth rate. The projected demand by the year 2025 is mind boggling (Hossain 1995), since the consumption rates will be faster than the population growth rates in all the major rice-growing Asian countries. Even in this scenario, the environmental-friendly strategies to grow more rice are of utmost significance, especially when the environmental costs of chemical fertilizers are accounted for.

The sustainability of rice production systems is intricately related to the chemical, physical, and biological properties of rice soils; these basic properties differ considerably between wet and dry land soils, tropical and temperate areas and, the soil surface and rhizosphere niche of the fields. Rice fields are important and unique in ecosystems because of the presence of diverse, biologically important microorganisms (Liesack *et al.* 2000). The paddy field ecosystem, therefore, comprises diverse habitats for microorganisms in time and space, such as aerobic/ anaerobic soil conditions, floodwater, rice roots, rice straw stubble and composted materials, which facilitate microbial growth and proliferation. The bacterial communities in paddy soils have been investigated continually using both cultivation-independent and cultivation-dependent molecular techniques. Recent advances in research on plant–microbe interactions revealed that plants are able to shape their microbiome, a critical factor to be considered for the use of microbial inoculants as biofertilizers or biocontrol agents.

The plant rhizosphere is known to be the preferred ecological niche for different types of soil microorganisms due to availability of nutrients, which in turn, is intimately related to the successful production of crops and sustenance of soil fertility (Bakker et al. 2013; Buckley and Schmidt 2001; Vessey 2003). Consortia of Plant Growth Promoting Rhizobacteria (PGPR) have been applied to various crops for improved seed germination, enhanced growth and yield. The soil microorganisms are associated with roots and as a consequence, the rice plants interact with these specific microbial communities, exhibiting a range of activities that include improved growth and yield (Yanni *et al.* 1997; Yanni and Dazzo 2010). Cyanobacteria represent an integral component of the flooded rice field ecosystems, and contribute immensely to the ecological functioning. The beneficial effects of these plant growth promoting bacteria including cyanobacteria have been attributed to biological nitrogen fixation and phytohormone production that lead to better root development and proliferation; these changes which are highly specific to certain plant species result in more efficient uptake of water and nutrients (Ashrafuzzaman *et al.* 2009; Mehnaz *et al.* 2001).

Cyanobacteria are the key players to sequester carbon and to improve the nutrient use efficiencies, besides their well-established role as nitrogen fixers (Mandal *et al*. 1998; Prasanna *et al.* 2012a, b). Several cyanobacterial strains, in combination with rhizospheric bacterial isolates, have been found promising to increase the growth and grain yield of rice as well as soil health. The N (nitrogen) savings in rice due to the cyanobacterial inoculation can be about 40-80 kg ha^{-1} (Prasanna *et al.* 2012a). Even an improved biofortification of grains in wheat has been reported (Rana *et al.* 2012). Through eliciting the plant defenses and entry into roots, the cyanobacterial strains can enhance growth and yields of rice and wheat crops (Jaiswal *et al.* 2008; Prasanna *et al*. 2008; Karthikeyan *et al*. 2009; Nain *et al.* 2010; Gopalakrishnan *et al.* 2010; Babu et al. 2014). Although they are obligate photoautotrophs, a sizeable community of cyanobacteria exists in the rice rhizosphere (Prasanna *et al.* 2009a, b). The present investigation was undertaken to analyze the rice plant microbiome (microbial communities in the soil, rhizosphere, roots, shoot and leaves) in the presence and absence of cyanobacterial inoculation under controlled conditions. Both the culture-based and culture-independent approaches, complemented by microscopic observations and soil nutrient / biochemical analyses were employed.

The physiological attributes of *Calothrix elenkinii* were examined before experimentation. Time course studies revealed that all the growth parameters (chlorophyll, ARA, IAA production) were highest in 3 or 4 weeks old cultures. ARA values ranged from 2.01-6.72 nmoles of C_2H_4 mL^{-1} h^{-1}. The activity of hydrolytic enzymes was highest in 3 weeks old cultures. Chitosanase activity was higher as compared to activity of other enzymes ranging from 0.75 to 0.1 IU mL^{-1} culture. An investigation on the microbiome (microbial communities in roots and shoots) in the presence and absence of cyanobacterial inoculation in rice seedlings under controlled conditions using water agar was undertaken. The rice cultivar cv. *Pusa Sugandh 5* (Pusa 2511) were used for experimentation. Root and shoot tissue extracts of 10 d old seedlings were used for enumerating the culturable microorganisms, using different media such as Yeast Extract Mannitol Agar (YEMA), Pikovskaya’s Agar, Soil Extract Agar (SEA), R2A Agar and Jensen’s Agar. Total count using different media was higher from root tissues than shoot tissues. Population densities of culturable microorganisms ranging from 10^6-10^9 CFU mL^{-1} were recorded in the root and shoot tissues in different types of nutrient media tested. Cyanobacterial inoculation led to 10 fold higher populations in the different media tested. Functional characterization of these isolates, five from nitrogen-free media (Yeast Extract Mannitol and Jensen’s media) and three isolates from the Pikovskaya medium revealed their potential for nitrogen fixation and phosphorus solubilisation, respectively. The enhanced nitrogen fixing potential of the *Calothrix* sp. inoculated rice plants can be the net result of selective enrichment of these diazotrophic bacteria. A total of 34 bacterial morphotypes (7 and 9 from inoculated root and shoots respectively and 9 each from root and shoot samples from control) were identified, from which 16 distinct morphotypes selected for Fatty Acid Methyl Ester (FAME) analyses. Only 60% of the isolates identified were in agreement with the library, when the similarity index value of $\geq$0.5 and the first choice were considered. Two isolates were identified with SI values of more than 0.8 while one of isolates could not be identified using this library. About 50% of the isolates were found to be of the Phylum Firmicutes, highlighting that they are most amenable to culturing. It is well known that plants cultivate their microbiome and reports on model systems such as *Arabidopsis thaliana* grown under controlled conditions or in natural soils also illustrated the significance of host genotype and soil type on the microbiome (Lundberg *et al.* 2012; Bulgarelli et al. 2012). Earlier report on the *Arabidopsis* roots showed that the composition of root-inhabiting microbial communities had a preferential colonization by members belonging to Proteobacteria, Bacteriodetes and Actinobacteria. The structural and functional diversity of rhizosphere of rice was analyzed by Lucas et al. (2014) and they observed that the dominant taxon was Proteobacteria, which is among the most documented and metabolically diverse group of soil microbes. A study on the diversity of endophytic bacteria present in seeds of a deepwater rice variety revealed the presence of many PGP endophytic bacteria which were able to produce indole acetic acid, reduced acetylene, mineral phosphate soloublization and showed specific immunological cross-reaction with anti-dinitrogenase reductase antibody. PCR fingerprinting data showed that one strain isolated from the surface sterilized seeds as well as the aerial parts of the seedlings of rice variety showed low cellulase and pectinase but relatively high ARA. Rice endophytic bacteria are known to originate from the outside environments, and invade the host through stomata, lenticels, wounds, areas of emergence of lateral roots and germinating radicles (Mano et al. 2007).

16S rDNA sequencing was taken up for selected isolates (from the set of 16 distinct morphotypes). About 50% of the isolates belonged to the Family Bacillaceae of the Phylum Firmicutes, which was in accordance with FAME analyses. Only one represented the Family Pseudomonadaceae, with the sequences similar to *Pseudomonas plecoglossicida* strain FPC951. This further emphasizes the pervasive nature of the members of the Gram-positive prokaryotes which are known to be comparatively stress resistant and long-range migrants, especially the Firmicutes and Actinobacteria. This enables them to inhabit diverse environments, such as the plant microbiome, besides extremophilic environments.

Analyses of the plant biometric parameters, along with metabolic functions of relevance to growth and nutrient acquisition in root and shoot tissues, revealed that the cyanobacterial inoculation brought about significant enhancement in plant growth, nitrogenase activity, indole acetic acid (IAA) production and activity of many defense enzymes. As this cyanobacterium *Calothrix elenkinii* is known to enter wheat roots

(Karthikeyan *et al.* 2009), scanning electron microscopic (SEM) observations of rice plants was done. The control plants showed the presence of rod-shaped cells in both the root and shoot tissues, whose numbers showed a significant increase in the *Calothrix* inoculated plants, besides the additional presence of short 2-3 celled filaments, presumably of *Calothrix* sp. in the root tissues. This may be attributed to the selective enrichment of useful bacteria and elimination of pathogenic microflora by the colonization of *Calothrix* sp., which is a promising PGP and biocontrol agent (Kumar *et al.* 2013a; Prasanna *et al.* 2014a, b) for various crops.

Based on the encouraging results of the water agar experiment, further experiments using pot experiments with sterile and unsterile soil was carried out under the controlled conditions of the National Phytotron Facility, IARI, New Delhi, for a better understanding of the interactions of soil and plant microbiome, with or without cyanobacterial inoculation. Evaluation of soil nutrient status and microbiological parameters were undertaken to assist in the interpretation of the data from culture-independent approaches. A significant enhancement in available nitrogen and phosphorus in both sterile and unsterile soil at 45 DAT, along with increased nitrogenase activity (acetylene reduction) and chlorophyll concentration of soils was recorded. The microbial community-based approaches - phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) profiling were employed.

Phospholipid fatty acids represent essential membrane components, with their polar head groups and ester-linked side chains exhibiting variation in compositions among members belonging to prokaryotes or eukaryotes. As they make up a relatively constant proportion of the organisms biomass, they provide a suitable index of total microbial biomass. As different taxonomic microbial groups synthesize PLFAs of distinct architecture, researchers have utilized them to study microbial community structure (Ferre *et al.* 2012). In the present study, the total PLFA(s) content of sterile soil was lower than that of unsterile soil and more than 30 distinct types of PLFA(s) including straight, branched, monounsaturated, polyunsaturated, dimethyl acetyl, and other mixed functional groups were obtained in both bulk and rhizosphere soils of rice. The individual fatty acid types of different PLFA profiles from soils on 15 DAT and 45 DAT clearly showed the alterations within the members of the major domains such as

Bacteria and Eukarya. Cyanobacterial inoculation led to decreases in the constituent members such as Gram-negative bacteria (67-70%), anaerobe (33-57%) and Gram-positive bacteria (11-49%). Not all the microbial guilds of identified by the PLFA markers responded to the cyanobacterial inoculation consistently in both the bulk and rhizosphere soils. Principal component analysis of these PLFA profiles showed that the inoculation with *Calothrix* brought about distinct temporal changes in the concentrations of individual PLFA(s), with some of them as 'biomarker PLFAs' for different microbial guilds (Gram positive- and Gram-negative bacteria, anaerobe, actinobacteria, fungi and eukaryotes). Reichardt *et al.* (1997) evaluated the shifts in microbial community structure as influenced by management practices and found a decrease on total microbial biomass at the time of flowering. The ratio of Gram-positive to Gram-negative bacteria was observed to be higher in flooded than in non-flooded soils. Ferre *et al.* (2012) observed that the Gram-negative bacteria were found to be most important for the characterization of aerobic conditions and for the decomposition of fresh residues. Investigations on plant–microbe interactions have revealed that plants or soil type can shape the total microbiome, as evidenced by the fact that different plant species exhibit specific microbial communities when grown the same soil or different soil type can bring about distinct variations in microbiome, even with the same host plant species (Lundberg *et al.* 2012; Bulgarelli *et al.* 2012). Pieffer *et al.* (2013) provided evidence for heritable variation in the rhizosphere microbial community composition of field grown maize inbreds.

DGGE profiles of microbial communities from rhizosphere, bulk soil and plant root and leaf samples and soil enrichment cultures, collected on 15 and 45 days after transplanting (DAT) provided evidence for the temporal changes on the composition of microbial communities due to cyanobacterial inoculation. Cluster analysis aided in illustrating the differences in the diversity of microbial communities of bulk and rhizosphere soils of rice with and without cyanobacterial inoculation. The similarity in the DGGE profiles of the sterile bulk soil (15DAT) and rhizosphere soil (45DAT after cyanobacterial inoculation) revealed the significant role of cyanobacteria on the diversity and abundance of different microbial communities. However, the cluster analyses showed that the microbiome of rice leaves of control plants and plants inoculated with cyanobacterium showed a similar pattern.

The present investigation, employing a combination of PLFA and DGGE profiles, plant biometric parameters and measures of soil nutrient status illustrated the important role of cyanobacteria in modulating the microbiome of rice. The cyanobacterial strain used in this investigation- *Calothrix elenkinii* is well documented for its role as a plant growth promoting and biocontrol agent for various crops, through the production of several metabolites, hydrolytic enzymes and phytohormones. Its ability to colonise rice and wheat roots and the rhizosphere makes it a promising agent for understanding its effect on soil and plant microbiome of rice. The positive effect of cyanobacterial inoculation on the rice plant growth can therefore be attributed to its ability to maintain the “beneficial core microbiome of rice," as a result of its growth-promoting and biocontrol properties. There is a definite need to identify the key microbial players in the core microbiome of rice for gaining a better understanding of the dynamics of microbial inoculation on plant and soil health.

7 SUMMARY AND CONCLUSIONS

Plants and microbes have evolved many beneficial and detrimental relationships which determine not only the plants' growth but also crop productivity and soil sustainability. Despite the availability of several reports on the interactions of microbial inoculants with crop plants, information is very scanty on their effect on the plant microbiome and the soil microbial communities. Rice is a globally important crop, with new information available on its genome sequences and proteomics providing valuable insights and better understanding of its responses to abiotic and biotic stress in the last decade. Cyanobacteria (or blue green algae, BGA) represent important inputs in the integrated nutrient management for this crop, and for maintenance of soil fertility of rice paddies. The present investigation was undertaken to investigate the changes in the structure and function of microbial communities in soil and the rice microbiome due to cyanobacterial inoculation, using both culturable and culture-independent approaches.

Based on the preliminary characterization of the physiological attributes of the cyanobacterial strain - *Calothrix elenkinii,* an investigation on the rice plant microbiome (microbial communities in roots and shoots) in the presence and absence of cyanobacterial inoculation in rice seedlings under controlled conditions in water agar was undertaken. The rice cultivar cv. *Pusa Sugandh 5* (Pusa 2511) was used. Employing cultural approaches, population densities ranging from 10^6-10^9 CFU/ml of culturable microorganisms were recorded in the root and shoot tissues in different types of nutrient media. Functional characterization of these isolates, five from nitrogen-free media (Yeast Extract Mannitol- and Jensen's media) and three isolates from the Pikovaskaya medium revealed their potential for nitrogen fixation and phosphorus solubilisation, respectively. Sixteen morphotypes were selected for profiling of fatty acid methyl esters (FAME) and 16S rDNA sequencing for their identification, which showed that about 50% of the isolates belonged to the Family Bacillaceae of the Phylum Firmicutes. The biometric analysis and biochemical characterization of root and shoot tissues revealed a significant enhancement in plant growth, nitrogenase activity, indole acetic acid (IAA) production and activity of many defense enzymes. The scanning electron microscopic (SEM) observations of rice plants revealed the presence of rod-shaped cells in both root and shoot tissues. However, the *Calothrix* inoculated plants exhibited a visible increase in the number of these cells and of short 2-3 celled filaments, presumably of *Calothrix* sp. in the root tissues.

In order to understand the interactions of soil and plant microbiome, with or without cyanobacterial inoculation, a pot experiment, using sterile and unsterile soil was carried out under the controlled conditions of the National Phytotron Facility, IARI, New Delhi. The culture-independent, microbial community-based approaches such as phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) profiling, complemented by evaluation of soil nutrient status and microbiological parameters were employed. The inoculation of *Calothrix elenkinii* resulted in significant enhancement in available nitrogen and phosphorus in both sterile and unsterile soil at 45 DAT, along with increased nitrogenase activity (acetylene reduction) and chlorophyll concentration of soils. Total PLFA(s) of sterile soil was less than that of unsterile soil and more than 30 distinct types of PLFA(s) including straight, branched, monounsaturated, polyunsaturated, dimethyl acetyl, and other mixed functional groups were obtained in both bulk and rhizosphere soils of rice. Principal component analysis of these PLFA profiles showed that inoculation with *Calothrix* brought about distinct temporal changes in the concentrations of individual PLFA(s), with some of them as ‘biomarker PLFAs’ for different microbial guilds (Gram positive- and Gram-negative bacteria, anaerobe, actinobacteria, fungi and eukaryotes).

The temporal changes on the composition of microbial communities due to cyanobacterial inoculation was studied by comparing the DGGE profiles of microbial communities from rhizosphere, bulk soil and plant root and leaf samples and soil enrichment cultures, collected on 15 and 45 days after transplanting (DAT). Numerical pattern (Cluster) analysis suggested the differences in the diversity of microbial communities of bulk and rhizosphere soils of rice with and without cyanobacterial inoculation. Both the bulk and rhizosphere soils of sterile (45 DAT) showed similar patterns. Interestingly, the DGGE profiles of the sterile bulk soil (15 DAT) and rhizosphere soil (45 DAT after cyanobacterial inoculation) showed similarity, evident from the distinct clusters. But, the cluster analyses showed that the microbiome of rice leaves of control plants and plants inoculated with cyanobacterium showed a similar pattern. These DGGE profiles illustrated the significant role of cyanobacteria in modulating the microbiome of rice

It can be concluded that positive effect of cyanobacterial inoculation on the rice plant growth is probably due to its role in maintaining the “beneficial core microbiome of rice." Future research should aim at characterizing the key microbial players in the core microbiome of rice and gaining a better understanding on the effect of cyanobacterial inoculation on the beneficial and pathogenic microflora.

ABSTRACT

Plants are full of microbes and phenotypic expression of most plants in nature is the product of highly co-regulated expression of genes both from plants and microbes. The present investigation was undertaken to analyse the effect of cyanobacterial inoculation on the structure and function of microbial communities in soil and the rice microbiome, using a combination of culturable and culture-independent approaches.

The cultural bacterial diversity of the rice plant microbiome (microbial communities in roots and shoots) was investigated in the presence and absence of cyanobacterial inoculation in rice seedlings, under controlled conditions in water agar using rice cultivar cv. *Pusa Sugandh 5* (Pusa 2511). Population densities ranging from 10^6-10^9 CFU/ml of culturable microorganisms were recorded in the root and shoot tissues in different types of nutrient media. Five and three isolates exhibited nitrogen fixation and phosphorus solubilisation, respectively. Sixteen morphotypes were selected for profiling of fatty acid methyl esters (FAME) and 16S rDNA sequencing, which showed that about 50% of the isolates belonged to the Family Bacillaceae of the Phylum Firmicutes. A significant enhancement in plant growth and physiological attributes were recorded which was complemented by SEM observations revealing an enhancement in rod-shaped cells in both root and shoot tissues of *Calothrix* inoculated plants, along with the presence of short 2-3 celled filaments, presumably of *Calothrix* sp.

In order to understand the interactions of soil and plant microbiome, with or without cyanobacterial inoculation, a pot experiment, using sterile and unsterile soil was carried out under the controlled conditions of the National Phytotron Facility, IARI, New Delhi. Culture-independent, microbial community-based approaches such as phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) profiling, complemented by evaluation of soil nutrient status and microbiological parameters were employed. Principal component analysis of these PLFA profiles showed that inoculation with *Calothrix* brought about distinct temporal changes in the concentrations of individual PLFA(s) and microbial guilds. DGGE based studies

illustrated the significant role of cyanobacteria in modulating the microbiome of rice. It can be concluded that positive effect of cyanobacterial inoculation on the rice plant growth is probably due to its role in maintaining the “beneficial core microbiome of rice."

सारांश

पौधों में सूक्ष्म जीवों की प्रचुरता है और प्रकृति में पौधों की आकारिकी का निर्धारण पादप एवं सूक्ष्म जीवों के जीनों से पूर्णतया सहनियमन द्वारा निर्धारित होता है। वर्तमान अध्ययन कल्चर योग्य एवं स्वतंत्र उपागम का प्रयोग करते हुए नील हरित शैवाल निवेशन का मृदा के सूक्ष्म जीव समूह एवं धान के सूक्ष्म जैव समुदाय के कार्य एवं संघटन पर प्रभाव का अध्ययन करना है।

धान के सूक्ष्म जैव समुदाय में जीवाणुवीय विविधीकरण का आकलन नील हरित शैवाल निवेशन की उपस्थिति एवं अनुपस्थिति में धान की प्रजाति पूसा सुगंध 5 (पूसा 2511) प्रजाति में नियंत्रित दशा में वाटर एगर को प्रयोग करते हुए किया गया। विभिन्न प्रकार के पौषण माध्यम में कल्चर योग्य सूक्ष्म जीवों की जनसंख्या जड़ एवं प्ररोह में 10^{-6} से 10^{-9} सी0 एफ0 यू0/मिली0 दर्ज की गई। आइसोलेट्स 5 एवं 3 में क्रमशः नत्रजन स्थिरीकरण एवं फॉस्फोरस घुलनशीलता पायी गई। फैटी एसिड मिथाईल एस्टर (एफ0 ए0 एम0 ई0) एवं 16 एस0 आर0 डी0 एन0 ए0 अनुक्रमण के लिए विभिन्न आकारिकी वाले 16 आइसोलेट्स को चयनित किया गया जिससे स्पष्ट हुआ कि लगभग 50 प्रतिशत आइसोलेट्स बैसीलेसी परिवार, फाईलम–फर्मीक्यूट से सम्बंधित है। पादप वृद्धि एवं कार्यिकी में सार्थक रूप से बढौतरी दर्ज की गई जो एस0 ई0 एम0 निरिक्षण द्वारा, कैलोथ्रिक्स द्वारा निवेशित पौधों में दण्डाकार कोशिका में बढत एवं 2–3 छोटी फीताकार कोशिका की उपस्थिति, जो कैलोथ्रिक्स प्रजाति की उपस्थिति से साबित होती है।

नील हरित शैवाल निवेशन की उपस्थिति एवं अनुपस्थिति में मृदा एवं पादप सूक्ष्म बायोम की अन्योन्य क्रिया को समझने के लिए निर्जमित एवं सामान्य मृदा को प्रयोग करते हुए एक गमला प्रयोग नियंत्रित दशा में राष्ट्रीय सुविधा भा0 कृ0 अनु0 सं0 में किया गया। कल्चर स्वतंत्र, सूक्ष्म जीव समुदाय आधारित उपागम जैसे फॉस्फोलिपिड़ फैटी एसिड़ (पी0 एल0 एफ0 ए0) एवं डिनेचरिंग ग्रेडिएंट जैल इलेक्ट्रोफोरेसिस (डी0 जी0 जी0 ई0) पार्श्व रूप रेखा का प्रयोग किया गया जो कि मृदा पौषण के मूल्यांकन एवं सूक्ष्म जैवीय मान के प्रयोग से साबित होता है। इन पी0 एल0 एफ0 ए0 की पार्श्व रेखा के प्रधान घटक के विश्लेषण से विदित है कि कैलोथ्रिक्स के निवेशन से व्यक्तिगत पी0 एल0 एफ0 ए0 के सांद्रण एवं सूक्ष्म जीव श्रेणी में स्पष्ट बदलाव होता है। डी0 जी0 जी0 ई0 आधारित अध्ययन से स्पष्ट है कि नील हरित शैवाल की धान के सूक्ष्म बायोम के अधिमिश्रण में सार्थक भूमिका है। इससे यह निष्कर्ष निकलता है कि नील हरित शैवाल का धान के पौधों पर धनात्मक प्रभाव संभवतः इसकी धान के लाभकारी सूक्ष्म बायोम के रखरखाव में भूमिका के कारण है।

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APPENDICES

Appendix 1: Composition of media

Soil Extract Agar

Ingredient	g L^{-1}
Soil Extract	15
Glucose	1
Dipotassium phosphate	0.5
Agar	15
pH	6.8±0.2

Yeast Extract Mannitol Agar

Ingredient	g L^{-1}
Mannitol	10
Yeast Extract	1
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium Chloride	0.1
Congo Red	0.025
Agar	20
pH	6.8±0.2

Jensen’s Medium

Ingredient	g L^{-1}
Sucrose	20
Dipotassium phosphate	1
Magnesium sulphate	0.5
Sodium Chloride	0.5
Ferrous Sulphate	0.10
Sodium Molybdate	0.005
Calcium Carbonate	2
Agar	15

YT Broth

Ingredient	g L^{-1}
Casein enzymic hydrolysate	16
Yeast Extract	10
Sodium Chloride	5

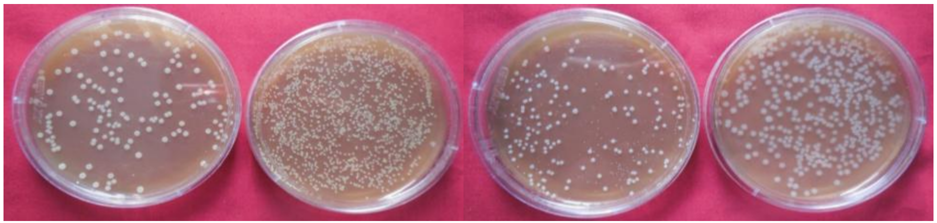
Pikovskaya’s Medium

Ingredient	g L^{-1}
Yeast Extract	0.5
Dextrose	10
Calcium Phosphate	5
Ammonium Sulphate	0.5
Potassium Chloride	0.2
Magnesium Sulphate	0.1
Manganese Sulphate	0.0001
Ferrous Sulphate	0.0001
Agar	18

R2A Agar

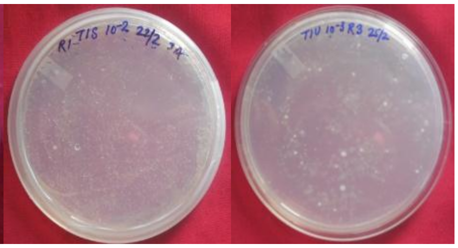
Ingredient	g L^{-1}
Casein Acid Hydrolysate	0.5
Dextrose	0.5
Proteose Peptone	0.5
Starch Soluble	0.5
Yeast Extract	0.5
Sodium Pyruvate	0.3
Dipotassium Phosphate	0.3
Magnesium Sulphate	0.024
Agar	18

Morphological diversity of culturable micro-organisms from rice microbiome

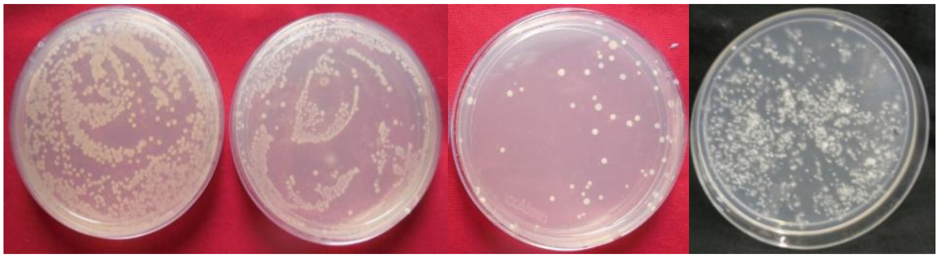


Soil Extract Agar

Yeast Extract Mannitol Agar



Jensen's Agar



R2A Agar

Morphotypes selected for FAME analysis