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ORIGINAL ARTICLE

Cyanobacterial diversity in the rhizosphere of rice and its ecological significance

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Abstract This investigation was undertaken to characterize the abundance and genera-wise diversity of cyanobacteria in the rice rhizosphere and nitrogen-fixing ability of the isolated strains. The cyanobacterial strains belonging to the genera *Nostoc* and *Anabaena* comprised 80% of the rhizosphere isolates, which were also efficient in enhancing the germination and growth of wheat seeds and exhibited significantly high protein accumulation and IAA production. Distinct profiles for the cyanobacterial strains were obtained on amplification with extended Hip 1 primer – HipTG, indicative of the diversity among these strains. Our investigation helped in identifying promising cyanobacterial isolates from the rhizosphere of rice, which can be utilized in developing efficient plant growth promoting cyanobacterial inoculants.

Keywords Cyanobacteria · Nitrogenase activity · Rhizosphere · PCR profiles

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Introduction

Cyanobacteria are known to cohabitate with rice, proliferating as floating assemblages on the soil-water surface and this has been exploited in agriculture through their specific inoculation as nitrogen supplementing biofertilizers in paddy fields, especially in several countries of South East Asia. A nitrogen accretion of 25–30 kg N/ha has been attributed to the activities of these ubiquitous organisms and correlated with the abundance of heterocystous forms as floating assemblages [1–3]. However, in recent years, an urgent need has felt to address inherent problems, which have limited their extensive exploitation, especially their poor establishment in diverse rice ecologies and soil types.

Cyanobacteria are known to inhabit a range of habitats, including plant surfaces [4–6], but this has not been adequately explored for improving their biofertilizing potential in agriculture and the rhizosphere is a relatively unexplored frontier. Watanabe and co-workers [7] observed that the associative nitrogen fixation of the rhizosphere was too low to meet the nitrogen demand of rice crop. However, Wada and co-workers [8] reported that the acetylene reducing activity of the rhizosphere of rice was higher than that of the surface soil layer or floodwater, emphasizing the significance of rhizospheric nitrogen fixation. A number of bacteria, especially the enterobacterial genera such as *Enterobacter*, *Serratia, Psuedomonas, Erwinia*, *Herbaspirillum*, *Gluconoacetobacter* are known to proliferate in the rice rhizosphere and play a significant role in soil and crop productivity [9], but cyanobacteria, have been mainly explored to-date as above-ground photosynthetic flora.

Attempts have been made to develop tight associations between cyanobacteria and rice plants, similar to legume–*Rhizobium* symbiosis [10, 11], so that a continuous

nutritional interaction throughout the period of crop growth is provided – which can help to overcome several problems related to their establishment. Nilsson and co-workers [11, 12] tested the efficiency of numerous symbiotic cyanobacterial isolates to associate with rice and found that under laboratory conditions, a number of them were successful in forming artificial associations. Cyanobacteria are also known to take up and assimilate organic compounds, which can play secondary, albeit important roles in their metabolism [13] and produce plant growth promoting substances [14].

Our investigation was directed towards evaluating the genera-wise diversity of cyanobacterial strains isolated from the rhizosphere of rice plants (grown under a range of agro-ecological conditions – including lowland, upland, deepwater and soils of diverse texture and pH at different locations in India), followed by characterization of these isolates for their growth attributes in light and dark, IAA production, nitrogen fixing ability and plant growth promoting ability on wheat seedlings under laboratory conditions. PCR based profiles of the rhizo-cyanobacterial isolates were also generated for the purpose of reliable identification.

Material and methods

Collection of plant samples and processing for isolation and enumeration of cyanobacteria: Healthy plants of different rice varieties at 45–50 DAT (days after transplantation) were uprooted with adhering rhizosphere soil, from diverse rice ecologies and geographic locations of India (Table 1). Excess soil was removed by gentle shaking and the adhering \soil was processed for enrichment cultures using nitrogen replete and deplete (with or without 1.5g l^{-1} $NaNO_3$) BG-11 medium [15]. Most probable number (MPN) technique was utilized for enumerating the nitrogen fixing and non-nitrogen fixing cyanobacterial populations in all the samples (including washed root bits of 1–3 cm). The cultures were incubated at 27 ± 1°C, 50–55 μmol photons m^{-2} s^{-1} light intensity and 16:8 L: D cycles.

Identification and purification of cyanobacteria: Standard plating/streaking techniques were used for isolation and purification of cyanobacterial strains [16]. Identification of the strains was done using the taxonomic keys of Desikachary [17] and designated by ID numbers in the figures and text. Generic diversity indices were calculated as given under statistical analyses.

Analytical procedures: The EC (electrical conductivity) and pH range was analyzed for all the soil samples (using soil: water = 1: 2.5) following the methodology outlined by Black [18].

For the biochemical analyses and ARA (acetylene reducing activity), uniform amount of inoculum was taken from rhizo-cyanobacterial cultures grown for several generations under optimal growth conditions. Dark grown cultures were also tested, but their growth was not sufficient for reliable estimation, in most of the strains. Nitrogenase activity was expressed as ARA (acetylene reduction activity). Commercially available standard ethylene was utilized for quantification and vials with equivalent volume of water served as controls. The ARA values were expressed per mg chlorophyll. 1 mL of gas mixture was removed from 15 mL glass vials with 5 mL cyanobacterial cultures, incubated under a gas mixture containing 10% acetylene and injected into preconditioned Nucon Model GLC 5500, housing a two-meter long Porapak R stainless steel column and 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 carrier gas. Standard ethylene gas (commercially available as a mixture with argon) was used for calibration and calculations [22]. All values presented are means of triplicate measurements.

The amount of proteins was determined according to Herbert et al. [19] with bovine serum albumin (BSA) as standard. IAA production was measured spectrophotometrically employing the method of Gordon and Weber [20]. Spectrophotometric estimation of the chlorophyll content of cyanobacterial cells was carried out following the method of Mackinney [21].

Table 1 Details of geographical locations of sampling sites in India along with physicochemical characteristics of soil

Locations	Latitude and Longitude (in degrees)	Rice Agroecology/Soil type	EC (ds/m) ± SD	pH ± SD
Aduthurai (Tamil Nadu)	11.00N, 78.00E	Irrigated lowland, alluvial	3.5 ± 0.1	7.5 ± 0.3
Hazaribagh (Bihar)	23.59N, 85.25E	Rainfed upland, light	3.0 ± 0.2	6.8 ± 0.1
Lucknow (Uttar Pradesh)	26.55N, 80.59E	Irrigated, saline-alkaline	4.7 ± 0.2	8.9 ± 0.2
Faizabad (Uttar Pradesh)	26.47N, 82.12E	Irrigated, lowland/upland	3.4 ± 0.2	7.9 ± 0.2
Ghagraghat (Uttar Pradesh)	27.30N, 81.20E	Deep water, alluvial	3.1 ± 0.3	8.4 ± 0.8
Indian Agricultural Research Institute (IARI) (New Delhi)	28.40 N, 77.20 E	Irrigated, sandy clay loam	3.8 ± 0.3	7.8 ± 0.2

Influence of rhizo-cyanobacterial isolates on seed germination: The seeds of wheat (*Triticum aestivum*, var. HD 2896) varieties were surface sterilized and placed on filter paper lined petriplates (5–6 seeds) and log phase cultures of a selected set of cyanobacterial strains (showing 100% germination) were added evenly on the filter paper. The petriplates were covered with aluminum foil and incubated at 28 ± 2°C for 2–3 days. The influence of these isolates on germination was made on a genera-wise basis and analyzed using SPSS package. Control treatments included those with sterile distilled water or BG 11 medium(only for experiments involving the measurement of length of coleoptiles and radical), instead of cyanobacterial cultures.

DNA fingerprints for the rhizo-cyanobacterial strains**:** Molecular profiling of the rhizo-cyanobacterial strains was carried out through PCR based amplification utilizing primers based on repetitive sequences and palindromes and employing whole cyanobacterial filaments as templates [23, 24]. These analyses were repeated atleast three times.

Statistical analyses: The data recorded in triplicate for the parameters in various strains were subjected to ANOVA (analysis of variance) in accordance with the experimental design (completely randomized block design) using SPSS (Statistical Package for the Social Sciences) to quantify and evaluate the source of variation. The diversity indices (Shannon's diversity index and Simpson's index of diversity) were calculated using the following formulae:

Shannon's Diversity Index = $-\sum$ (ni/N) ln (ni/N)

Where, ni = the percentage of a particular as type of BGA; N= 100

Simpson's Index of Diversity = 1–D

where $$D = \frac{\sum n(n-1)}{N(N-1)}$$

n = the total number of organisms of a particular species

N = the total number of organisms of all species

Table 2 Enumeration of cyanobacterial populations, their genera-wise distribution and diversity indices for the various locations and rice cultivars

Geographic locations and varieties	Genera[1]								MPN/g soil (x 10^4)		Diversity indices	
	Ana	Nos	Cal	Hap	Wes	Scy	Ph	Os	+N	-N	Shannon diversity index (*H*)	Simpson's index of diversity (1-D)
Aduthurai (Tamil Nadu)												
ADRH –1	5	5	-	-	-	-	1	-	100.00	1.00	0.936	0.637
Pusa 44	3	1	-	-	-	-	-	-	10.00	9.10	0.561	0.500
Hazaribagh (Bihar)												
Anjali	1	2	-	2	3	-	-	-	1.00	12.00	1.319	0.822
Hazaridhan	2	-	1	-	1	-	1	-	5.40	1.00	1.332	0.900
Lucknow (Uttar Pradesh)												
CSRC 13	2	2	1	1	-	-	-	-	0.002	0.068	1.334	0.867
CSRC 14	3	-	-	-	-	1	-	-	0.092	0.017	0.562	0.500
Faizabad (Uttar Pradesh)												
Sarju 52	4	2	-	-	-	-	-	-	7.00	1.00	0.634	0.533
NDR 3026	-	2	2	1	-	-	-	1	0.019	0.0049	1.334	0.867
Ghagraghat (Uttar Pradesh)												
Madhukar	1	2	-	1	-	-	-	-	8.24	0.0001	1.041	0.833
Jalpriya	2	2	-	-	-	-	-	-	9.31	0.0002	0.694	0.667
Badavarodhi	3	3	-	1	-	-	-	-	12.1	0.0026	1.004	0.714
IARI (New Delhi)												
Pusa Basmati	2	4	-	1	-	-	-	-	0.02	0.07	0.956	0.667

[1]Ana: *Anabaena;* Nos: *Nostoc;* Cal: *Calothrix;* Hap: *Hapalosiphon,* Wes: *Westiellopsis;* Scy: *Scytonema;* Ph: *Phormidium;* Os: *Oscillatoria*

Results and discussion

Cyanobacteria are known to be an integral component of waterlogged rice fields, which supply around 86% of the global requirement of rice. The favorable balance of soil N of wetlands, wherein rice can be grown on the same land without any fertilizers and without any detectable decline in yield, attests to the significance of cyanobacterial nitrogen fixation. Also, indirect evidence for its potentiality is available through demonstrations of enhanced crop growth and yields [25–28]. The present investigation was aimed towards a less investigated domain of cyanobacteria – the rhizosphere, as a means of identifying isolates which can establish and proliferate in soil and prove better competitors when applied as biofertilizer consortia.

The physicochemical environment of the sample collection sites was diverse in terms of EC and pH (Table 1). Hazaribagh soil samples were neutral in their pH, with a moderate EC of 3.0 d Sm^{-1}. The saline soil samples of Lucknow exhibited a high EC of 4.7 d Sm^{-1} and a highly alkaline pH of 8.9. Faizabad soil samples also exhibited a slightly alkaline pH of 7.8, while the other two samples exhibited neutral pH and moderate EC. Although cyanobacteria are ubiquitous in their distribution, it is well established that they prefer a neutral – slightly alkaline pH.

Enumeration and isolation of cyanobacterial populations in the rhizosphere soil and root bits using MPN technique, revealed a wide range in the populations (Table 2). Despite the inherent deficiency of MPN technique, related to non-inclusion of uncultivable strains, it remains one of the widely used methods for enumeration about viable populations in soil which have been refined to improve their utility [29]. Highest populations of 9.1×10^4 and 1×10^6 were recorded in Aduthurai rice cultivars in nitrogen deficient and supplemented media. The rice cultivar CSRC 13 from Lucknow, grown under slightly alkaline and saline conditions, exhibited lowest population counts in nitrogen enriched medium, while the deepwater cultivars from Ghagraghat recorded lowest count in nitrogen deficient medium. In the present investigation, the cyanobacteria isolated from the two cultivars CSRC-13 and CSRC-14, from the saline soils of Lucknow showed characteristic mucilaginous sheaths and in general, exhibited low generic diversity, besides low population counts. Besides the significant effect of soil physicochemical characteristics, it is well understood that the crop plant exerts an influence on the qualitative and quantitative nature of microbial populations, including cyanobacteria, in the rhizosphere. In the two cultivars – *Anjali* and *Hazaridhan* from Hazaribagh, growing in a similar ecological habitat, quantitative and quantitative differences were observed in their rhizo-cyanobacterial populations. This is indicative of the influence of specific cultivar and its root exudates on the type and number of cyanobacterial genera. The root bits and soil samples collected from the rhizosphere of cultivar *Madhukar*, grown in Ghagraghat exhibited very high population of cyanobacteria in nitrogen enriched medium, but very low numbers in medium devoid of combined nitrogen as against the other varieties grown in this area. Comparatively, all the deepwater rice cultivars from Ghagraghat exhibited very low populations in nitrogen deplete medium, but very high counts in nitrogen supplemented medium. Interestingly, maximum number of *Nostoc* strains were isolated from these varieties *i.e* abundance of a single genus or low generic diversity. Therefore, cyanobacterial abundance in these samples could also be attributed to the different soil characteristics and rice cultivars utilized. Chemical analyses of the root exudates from these rice cultivars may provide further clues to the specificity and abundance of cyanobacterial genera and the nature of associations.

Heterocystous forms belonging to *Anabaena* and *Nostoc* were observed to be the dominant genera in the rhizosphere. The genera-wise diversity in rhizosphere of the rice cultivars at each of the locations is given in Table 2. A total of 28 isolates belonging to genus *Anabaena* and 25 of *Nostoc* were obtained. *Hapalosiphon*, *Westiellopsis* and *Calothrix* were the other heterocystous genera isolated from the rhizosphere. Among the non-heterocystous cyanobacteria, *Oscillatoria* and *Phormidium* occurred in the rhizosphere, but not in all rice varieties. *Scytonema,* was observed to predominate in saline soil samples from Lucknow. *Nostoc* and *Anabaena* were the dominant forms in all the samples, especially in Aduthurai and Ghagraghat. Hazaribagh samples recorded a high number of *Westiellopsis* and *Hapalosiphon* isolates. Of the 22 cyanobacterial strains were isolated from Ghagraghat, 11 were *Nostoc* and 8 *Anabaena* strains. Green algae, belonging to the genus *Chlorococcum,* and other unicellular/colonial genera were also recorded in most of the samples.

Earlier studies on cyanobacterial diversity of rice fields at the Indian Agricultural Research Institute have shown the predominance of genera – *Nostoc, Anabaena* and *Phormidium*, irrespective of chemical/biofertilizers treatments and stage of crop growth [23, 24]. This indicates that these genera are highly competitive, whether as floating assemblages or in the rhizosphere. *Nostoc* is known to be one of the most versatile diazotrophic cyanobacterial genera, observed in free-living state and in symbiotic associations with a wide range of fungi, bryophytes, gymnosperms and angiosperms. The members of this genus are capable of a variety of modes of C and N nutrition, which adapts them to diverse ecological habitats and the biotrophic transfer of

fixed N to the hosts [10]. Song et al. [28] used semi nested PCR and DGGE to evaluate the cyanobacterial diversity and seasonal changes in a selected rice field, and identified 24 phylotypes, including *Nostoc* and *Phormidium*, some of which were specific to the stage of sampling (i.e. rice growth season).

In the present study, generic richness or the number of genera per unit area/volume varied with respect to varieties within a location, and among locations. Rhizosphere samples from rice cultivars grown at Aduthurai exhibited low generic richness i.e. only two genera – *Anabaena* and *Nostoc* were observed. On the other hand, from the cultivar *Anjali* (Hazaribagh) strains belonging to four different genera were isolated from its rhizosphere samples. The quantification of diversity, in terms of evenness is a measure of the relative abundance of the different species/genera making up the richness of an area, and provides a measure of the equality in abundance of species, or genera as evaluated in this study. It was observed that the Aduthurai rice cultivars – *ADRH-1* and *Pusa 44, Sarju 52* (from Faizabad), *CSRC 14* (from Lucknow) and *Jalpriya* (Ghagraghat) had less evenness, as only two genera were present. Highest Shannon's diversity indices were recorded for *CSRC 13* (Lucknow) and *NDR 3026* (Faizabad) followed by cultivars *Anjali* and *Hazaridhan* (Hazaribagh). *Pusa 44* (Aduthurai) and *CSRC 14* (Lucknow) exhibited lowest *H* as only four strains; comprising two genera were isolated from its rhizosphere samples. Shannon's index was indicative of extensive diversity within the cultivars in all locations and emphasized the significant role of rice cultivar in generating cyanobacterial diversity. On analyzing the genera-wise diversity using Simpson's index of Diversity (1-D), which takes into account both richness and evenness, highest values were recorded from cultivar *Hazaridhan* (Hazaribagh), followed by *NDR 3026* and *CSRC 13*.

Gantar et al. [5] carried out colonization studies of cyanobacteria with wheat seedlings and observed that they contributed significantly to the nitrogen economy of wheat plants. In the present investigation, high diversity was observed with respect to the nitrogenase activity of the 69 heterocystous isolates from the rhizosphere, incubated under light (Fig. 1). All the isolates have been designated by ID numbers (1–69), which include 1–14 from Aduthurai, 15–26 from Hazaribagh, 27–36 from Lucknow, 37–47 from Faizabad and 48–69 from Ghagraghat. Specific activity of nitrogenase ranged from 4.06 (ID No.62) to 844–72 (ID No. 67) ηmoles C_2H_4 mg proteins^{-1} h^{-1}. Highest values were recorded in the rhizosphere isolate of *Nostoc* from the deepwater variety *Badavarodhi* from Ghagraghat. Comparatively, the *Anabaena* and *Nostoc* isolates exhibited high nitrogenase activity.

The ability of cyanobacteria to produce biologically active growth promoting substances such as the phytohormone IAA has been demonstrated in very few strains [14, 31]. A set of twenty strains exhibiting high ARA was further evaluated in terms of protein accumulation in light and dark (+ 0.5% glucose) and IAA production. Diversity was observed in terms of protein accumulation and IAA production, under light and dark conditions, in the selected set of strains. Protein accumulation, was very high under both these conditions, and strains having ID numbers 45 and 13 exhibited very high values when incubated in dark (Fig. 2). Highest

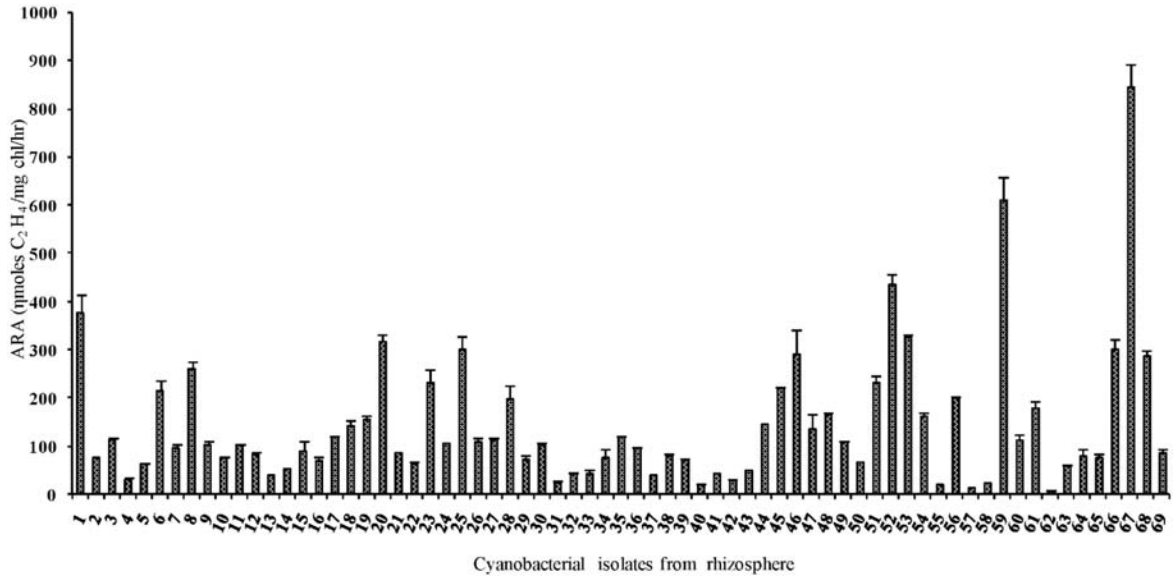

Fig. 1 Acetylene reducing activity (ARA), measured as an index of nitrogenase activity of the different rhizo-cyanobacterial strains. Vertical bars on columns represent SD ($n = 3$).

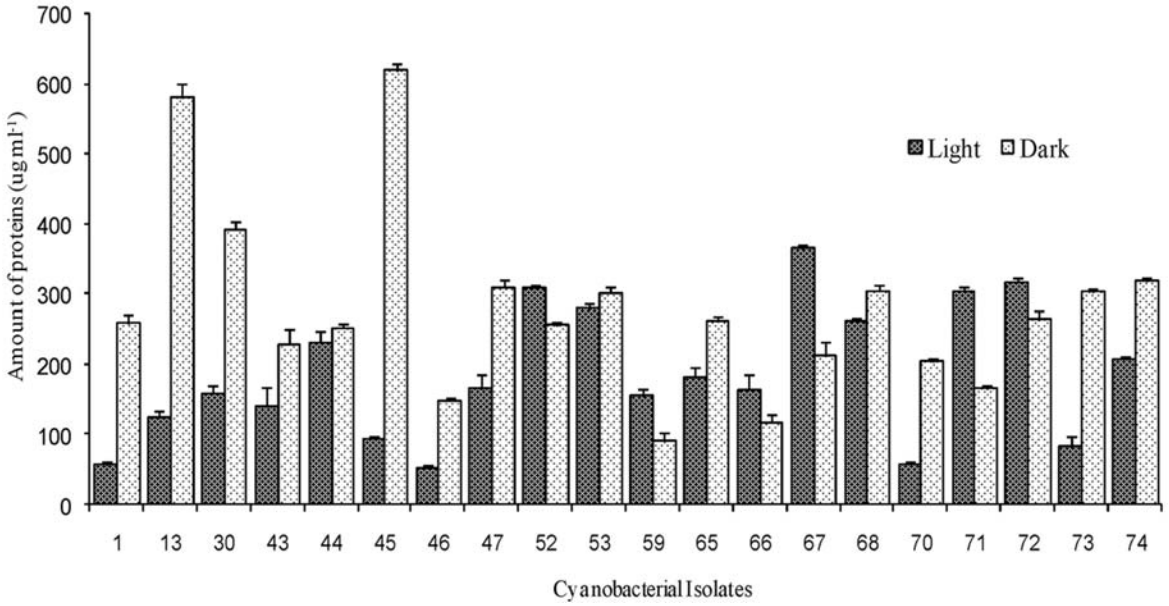

Fig. 2. Protein accumulation in a selected set of rhizo-cyanobacterial strains, in light and dark grown cultures. Vertical bars on columns represent SD ($n = 3$).

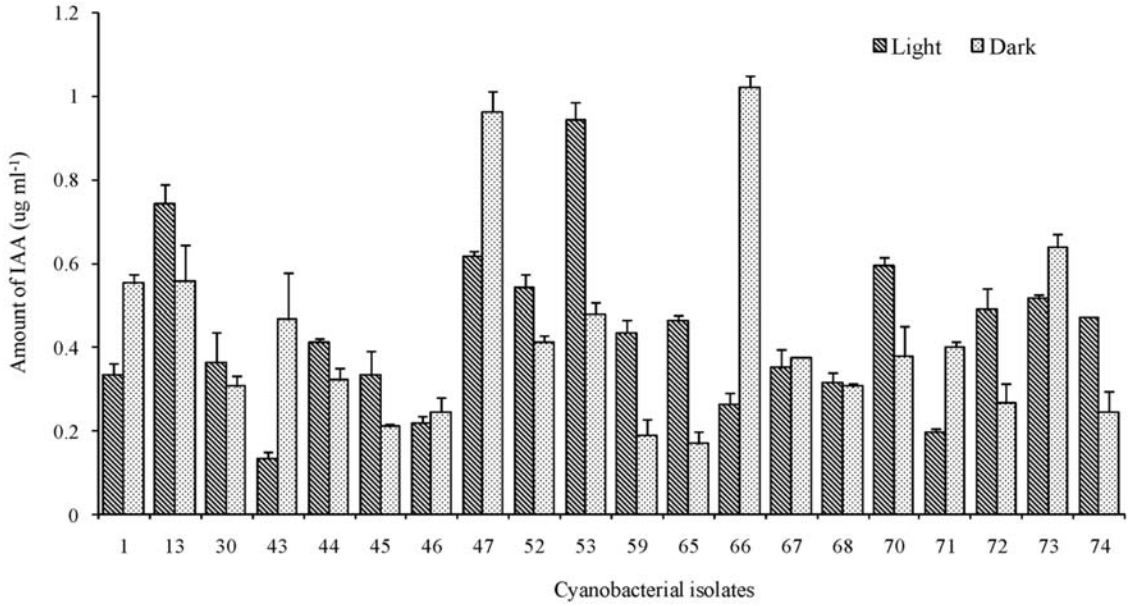

Fig. 3. IAA production in a selected set of rhizo-cyanobacterial strains, in light and dark grown cultures. Vertical bars on columns represent SD ($n = 3$).

IAA production was recorded in strain numbers 66 and 47 incubated in dark while light-incubated strains – 53 and 13 also exhibited high values (Fig. 3) indicating that these rhizosphere isolates are in growing state and produce/excrete metabolites into their immediate environment.

The influence of the cyanobacterial cultures on seed germination and length of radicle and coleoptile of wheat seedlings was significant, especially on wheat seeds (Fig. 4). Highest coleoptile length was observed in strain number 66, followed by 47, which also produced maximum IAA in dark. The cyanobacterial isolates from rice rhizosphere exhibiting plant growth promoting ability on wheat is significant, as such strains can be deployed where rice –wheat cropping sequence is undertaken.

Pedurand and Reynaud [31] reported that among a set of cyanobacterial strains isolated from dry tropical Africa soils (mainly from rice fields), 70% exhibited a negative effect on germination of rice seeds. However, in the present study,

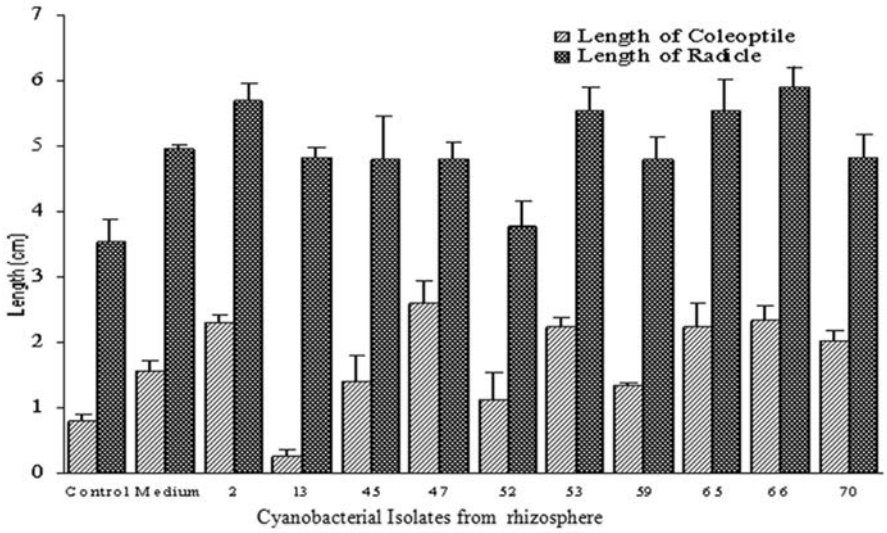

Fig. 4. Influence of rhizo-cyanobacterial strains on the germination of wheat seedlings. Vertical bars on columns represent SD ($n = 3$).

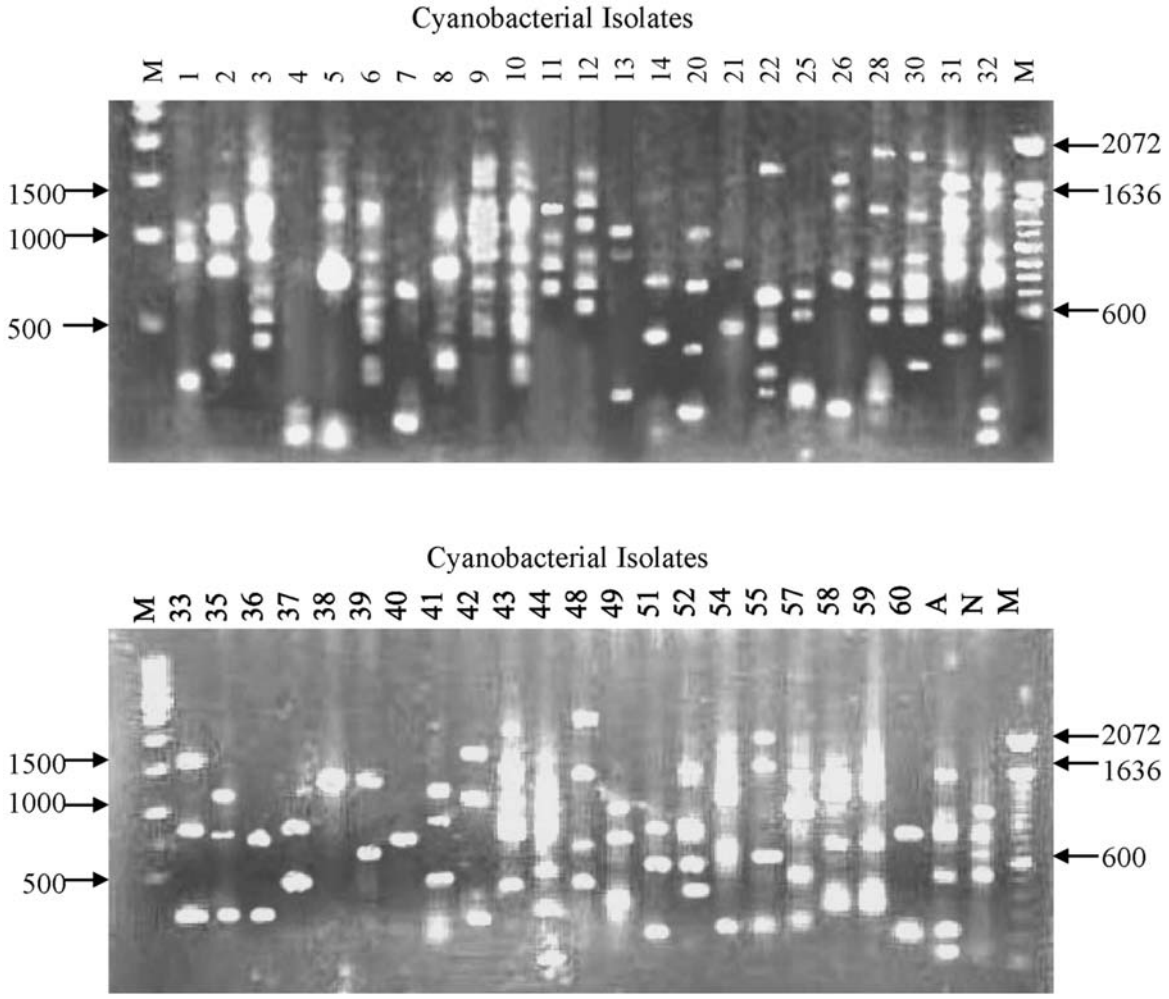

Fig. 5. PCR fingerprint patterns of intact filaments of rhizosphere isolates belonging to cyanobacterial genera – *Anabaena* and *Nostoc* from diverse locations. ID Numbers (as given in text) a. 1-14 (Aduthurai); 20,21,25,26 (Hazaribagh); 25,30,31,32 (Lucknow); b. 33,35,36 (Lucknow); 37-44 (Faizabad); 48,49,51,52,54, 55,57,58,59,60 (Ghagraghat); A (*Anabaena* sp. PCC 7108), N (*Nostoc* sp. PCC 7107).

only 10% of the strains did not bring about 100% germination of wheat seeds suggesting that 90% of these strains can be safely utilized as inoculants.

In order to facilitate identification of these isolates and depict the genetic diversity, DNA fingerprinting was carried out using a set of primers based on repeat sequences/palindromes, which have been employed for molecular profiling work on cyanobacterial symbionts of *Azolla*, *Cycas* and *Gunnera* [12]. In the present investigation, an extended Hip 1 primer – HipTG, which was utilized by Zheng et al. [24] to analyse the diversity among symbiotic cyanobacteria associated with cycads, was employed. Distinct fingerprints were generated for a selected set of rhizo-cyanobacterial strains (Fig. 5). The results showed that the profiles of these *Anabaena* and *Nostoc* isolates were not similar to that of free-living strains (*Nostoc* sp. PCC 7107, *Anabaena* sp. PCC 7108).

The general belief that cyanobacteria are obligate photoautrophs has been, perhaps, the major reason for the paucity of information available on these microorganisms, in this niche. However, the results of the occurrence and metabolic activity in the rhizosphere zone suggest that they can prove to be better competitors when deployed as soil inoculants or as seedling inoculants.

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