



Characterizing cyanobacteria from Punjab rice varieties for growth promotion

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ABSTRACT

Cyanobacterial strains were isolated, and identified from rhizospheric soil and plant parts of rice varieties from Ludhiana, Punjab during *kharif* 2018. Out of the total 18 strains, 16 were heterocystous and 2 were non-heterocystous. Seven were isolated from rice variety PB-1121 while 11 were from variety Parman-126. These belonged to four genera, *Nostoc* (13), *Anabaena* and *Phormidium* (2 each); and *Trichormus* (one). These were characterized for cell dry weight, pigments and total soluble proteins which showed a significant variation. Pigments ranged from 1.51 µg/ml to 11.63 µg/ml (chlorophyll), 0.41 µg/ml to 11.06 µg/ml (carotenoids), 1.13 µg/ml to 26.28 µg/ml (phycocyanin), 0.15 µg/ml to 33.23 µg/ml (phycoerythrin), 0.16 µg/ml to 11.18 µg/ml (allophycocyanin). Total phycobili proteins varied from a highest of 54.31 µg/ml to the lowest of 2.95 µg/ml, whereas, total soluble proteins ranged as 0.29 mg/ml to 0.70 mg/ml. Nitrogenase activity was measured as Acetylene Reduction Assay and this parameter also varied amongst the strains. Extracellular ammonia release varied as lowest of 11.10 µmole NH₄⁺/ml to the highest of 229.70 µmole NH₄⁺/ml, whereas, indole acetic acid production ranged from 7.45 µg/ml to 66.00 µg/ml. On the basis of efficient plant growth promoting parameters, these cyanobacterial strains can be studied for their possible positive influence on the performance of rice crop, which may, in turn, help in nitrogen economy after testing under pot culture and field evaluations as per required procedure and guidelines.

Keywords: Characterization, Cyanobacteria, Isolation, Plant growth promoting attributes, Rice

Cyanobacteria exhibit oxygenic photosynthesis and many are diazotrophic, which allows them to grow in absence of combined nitrogen (Castenholz 2001), hence, these play an important role in rice field ecosystems (Venkataraman 1981). Pigments such as chlorophyll *a*, carotenoids and phycobiliproteins are light harvesting components (Takaichi *et al.* 2009) and phycobili proteins account for 20% of total dry weight (Bogorad 1975). These organisms can help in maintenance and build-up of soil fertility, therefore, are considered as natural biofertilizer (Song *et al.* 2005). Added biomass in the soil can improve the organic matter, improves the fertility of soil following death and decomposition, and controls weed growth (Saadatnia and Riahi 2009). Cyanobacterial growth can also increase the availability of soil phosphates by excreting organic acids (Wilson 2006).

Cyanobacteria can be present as free living or as associated in rice field ecosystem. The biologically fixed nitrogen can enhance nitrogen economy of crops and reduce the requirement for nitrogenous fertilizers (Sturz and Nowak 2000). Their diversity study showed that *Nostoc* and *Anabaena* comprise 80% of rhizospheric population of rice plant from different agroecologies of India (Prasanna *et al.* 2009). Species of *Nostoc*, *Anabaena*, *Tolypothrix*, *Aulosira*, *Cylindrospermum*, *Scytonema*, *Westiellopsis* and others which are widespread in Indian rice fields are known to contribute significantly to fertility (Venkataraman 1981, Choudhary 2011). Cyanobacteria can also interact with cells of the host through secreted metabolites and plant-growth-promoting (PGP) compounds like phytohormones, vitamins and amino acids etc. (Hardoim *et al.* 2011). Auxin production has been reported in *Chroococcidiopsis* SM-04, *Synechocystis* SM-10, *Leptolyngbya* SM-13, and *Phormidium* SM-14 from rhizosphere and soil surface of wheat, rice and maize (Mazhar *et al.* 2013). Further, several strains of *Anabaena* have been reported to produce IAA (mostly in the range of 1–3 µg/ml) in the nitrogen free BG-11 medium devoid of tryptophan (Prasanna *et al.* 2008a). Most of the known cyanobacterial plant associations may involve mutual exchange of nutrients, especially related to the fixation of carbon or nitrogen (Karthikeyan *et al.*

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2009). In view of this, present study was undertaken to decipher cyanobacterial diversity and abundance present in rhizospheric soil and plant parts of selected rice varieties of Punjab, India.

MATERIALS AND METHODS

Experimental site and plant sampling: Four to five plants of rice varieties, viz. PB-1121 and Parman-126 were collected during 30-45 days after transplantation during *kharif* 2018 in a zigzag sampling pattern from fields Punjab Agricultural University, Ludhiana. Simultaneously, rhizospheric soil was also collected and pooled from each variety. Both plant and soil samples were brought to the laboratory of CCUBGA, ICAR-IARI for the isolation of cyanobacteria and their characterization.

Isolation, purification, identification and maintenance of cyanobacterial strains: Serial dilutions of rhizospheric soil were prepared for isolation of cyanobacteria. From 10^{-2} dilution, 500 μ l was spread plated on agar based BG-11 (+N and -N) medium (Stanier *et al.* 1971). The 1 g of plant parts (leaves, stem and roots) was surface sterilized using 70% ethanol and 0.02% HgCl_2 for 1 min and 2 min followed by washing thoroughly with sterilized water. These were macerated in sterilized mortar pestle and inoculated in BG-11 (+N and -N) liquid medium for isolation of endophytic cyanobacteria. The plates and flasks were incubated at $28 \pm 2^\circ\text{C}$ temperature, 50-55 $\mu\text{mole photons/m}^2/\text{slight intensity}$ and 16:8 Light: Dark cycles for 20-25 days under standard cultural conditions. The plates having cyanobacterial growth were examined critically and the isolates were selected on the basis of size, color, shape and texture of colonies. The similar looking individual colonies were picked and examined microscopically for purity. The isolates were purified by repeated sub culturing, plating and streaking. For the isolation from plant parts, 200 μ l of suspension showing growth was taken from the flasks and was spread plated on respective BG-11 (+N or -N) medium. Colonies were picked up and strains were purified in a similar manner as described. Monoclonal strains were kept under standardized conditions to limit morphological and morphometric variations. Isolated and purified cyanobacterial strains were identified microscopically using Fluorescence Microscope (Classic FL, Dewinter) based upon the keys given in Desikachary (1959) and Komarek and Anagnostidis (2005). Discreet colonies from rhizospheric soil and plant parts were maintained in respective fresh medium and used for study at exponential phase (14th day) of growth.

Growth parameters: Cell dry weight (mg/ml) was determined by taking a known volume of homogenized cyanobacterial suspension, filtered on a previously dried pre-weighed Whatman No. 42 filter paper, dried at 60°C for 3-5 h and cooled till constant weight (Sorokin 1973). For estimation of pigments, hot methanol extraction method was utilized for the estimation of chlorophyll by taking absorbance at 650 and 665 nm against 95% methanol as blank (Lichtenthaler and Buschmann 2001). Cold extraction process using acetone was used to estimate total carotenoids

by taking absorbance of supernatant at 450 nm using 85% acetone as blank (Jensen 1978). For phycobiliproteins, the pellet from suspension was taken in phosphate buffer (0.05 M) and was subjected to repeated freezing and thawing till complete extraction. The absorbance of supernatant was measured at 562, 615 and 652 nm against phosphate buffer as blank. The amount of phyco-biliproteins (Phycocyanin, PC; Allophycocyanin, APC; and Phycoerythrin, PE) was calculated (Bennett and Bogorad 1973). The method of Lowry *et al.* (1951) was followed for the estimation of total soluble proteins. The absorbance was recorded at 650 nm against blank. The concentration of total soluble proteins was estimated using a standard curve of BSA ranging from 10-160 $\mu\text{g/ml}$.

Plant growth promoting attributes: Quantification of ethylene from acetylene was taken as an index of nitrogenase activity as a plant growth promoting property. Acetylene reduction activity (ARA) was measured in Gas Chromatograph (Model; Shimadzu) during exponential phase using homogenized suspension incubated with acetylene gas. Ethylene was estimated against standard ethylene and ARA was expressed as $\text{nmole C}_2\text{H}_4/\text{mg chl/h}$ (Hardy *et al.* 1973). Extracellular ammonia was estimated in the filtrate by spectrophotometric absorbance at 640 nm and expressed as $\mu\text{mole NH}_4^+/\text{ml}$ (Solorzano 1969). For Indole Acetic Acid, the cyanobacterial isolates were grown in the presence of L-tryptophan at concentration of 1000 $\mu\text{g/ml}$ in the growing medium. The amount of IAA in culture filtrate was determined spectro-photometrically by measuring intensity of pink color at 530 nm using standard curve of IAA and was expressed as $\mu\text{g/ml}$ (Gordon and Weber 1951).

Statistical analysis: The growth parameters and PGP related data was analyzed for one factor analysis of variance using MS-Excel (ANOVA) table and all the values were mean of three replications.

RESULTS AND DISCUSSION

Isolation, purification and identification of cyanobacterial strains: On the basis of microscopic observations, a total of 18 cyanobacterial strains were isolated and purified from rhizospheric soil and plant parts of rice varieties of Punjab. Out of these, 7 were from variety PB-1121 and 11 were from Parman-126. The strains of PB-1121 were designated as P1 to P7, whereas, strains from Parman-126 were designated from P8 to P18. All the isolates were heterocystous except P7 and P18 which were non-heterocystous. These strains belonged to four genera namely, *Nostoc*, *Anabaena*, *Phormidium* and *Trichormus*. From variety, PB-1121, 5 strains, viz. *Nostoc ellipsosporum* (P1), *Anabaena* sp. (P4), *Trichormus anomalus* (P5), *Nostoc cellipsosporum* (P6) and *Phormidium* sp. (P7) were from rhizospheric soil, whereas, 2 strains of *Nostoc* sp. (*Nostoc cellipsosporum* P2 and *Nostoc ellipsosporum* P3) were from root. However, from variety Parman-126, 3 strains, *Nostoc* sp. (P15), *Nostoc cellipsosporum* (P16) and *Phormidium* sp. (P18) were from rhizospheric soil, one strain of *Nostoc* sp. (P13) was from

root, 3 strains *Nostoc ellipsosporum* (P9), *Anabaena* sp. (P10) and *Nostoc ellipsosporum* (P14) were from stem, whereas, 4 strains (*Nostoc carneum*, P8; *Nostoc* sp., P11; *Nostoc* sp., P12 and *Nostoc* sp., P17) were from leaves.

Growth parameters: Cell dry weight (mg/ml) showed marked difference amongst strains and was lowest in strain P6 (0.21 mg/ml) and highest in strain P5 (2.05 mg/ml). In addition to chlorophyll as a photosynthetic pigment, cyanobacteria are attractive source of carotenoids, phycobiliproteins and other vital secondary metabolites (Moreno *et al.* 1998). Chlorophyll showed significant variability amongst the strains from two varieties. The values ranged from lowest of 1.51 $\mu\text{g/ml}$ to the highest of 11.63 $\mu\text{g/ml}$. Strain P11 showed highest chlorophyll, whereas, strain P18 recorded lowest chlorophyll and these were isolated from leaf and rhizosphere of variety Parman-126 (Fig. 1). Similarly, chlorophyll ranged from 0.17 $\mu\text{g/ml}$ to 4.89 $\mu\text{g/ml}$ amongst 24 strains of *Anabaena* (Nayak *et al.* 2007). Prasanna *et al.* (2006) also examined different strains of *Anabaena* in relation to chlorophyll which showed a wide variation from 0.11 $\mu\text{g/ml}$ to 3.40 $\mu\text{g/ml}$.

Carotenoids and phycobili proteins function as accessory pigments and play significant role in light harvesting for photosynthesis. Besides that, carotenoids quench singlet and triplet states of chlorophyll *a*, providing protection from excessive light (Jeffrey *et al.* 1997). Amongst selected strains of *Anabaena*, carotenoids ranged from 0.4 $\mu\text{g/ml}$ to 21.63 $\mu\text{g/ml}$ (Prasanna *et al.* 2006). In the present study, the strain P12 showed highest carotenoid of 11.06 $\mu\text{g/ml}$, while, strain P13 exhibited lowest carotenoid of 0.41 $\mu\text{g/ml}$ (Fig 1). Total phycobili proteins were highest (54.31 $\mu\text{g/ml}$) in strain P12 having second highest PC and PE.

Phycocyanin (PC) varied from lowest of 1.13 $\mu\text{g/ml}$ in P4 (PB-1121) to highest of 26.28 $\mu\text{g/ml}$ in P15 (Parman-126). On the other hand, P17 (Parman-126) recorded highest (33.23 $\mu\text{g/ml}$) phycoerythrin and it was lowest (0.15 $\mu\text{g/ml}$) in P5 (PB-1121). Strain P12 from Parman-126 showed phycoerythrin of 31.70 $\mu\text{g/ml}$ and this also showed highest carotenoids. The lowest allophycocyanin (0.16 $\mu\text{g/ml}$) was recorded in P5, whereas, highest (11.18 $\mu\text{g/ml}$) was in P8 followed by P2 (7.29 $\mu\text{g/ml}$) (Fig 2). Nayak *et al.* (2007) also reported variation in percent phycobili proteins amongst *Anabaena* strains and found that strain CCC 160 produced highest (52.86%), whereas strain CCC83 recorded lowest (6.84%) phycobili proteins. Further, most of the strains were phycocyanin rich, whereas, two were phycoerythrin rich. Bryant (1982) reported the production of PC in all naturally occurring cyanobacteria, whereas, PE was not reported always. Our results are consistent with the Bryant's work as PC rich strains were common and phycoerythrin content was negligible amongst strains P1, P2, and P5. However, some such as P8, P9, P12, and P17 showed high PE. Simeunović *et al.* (2013) observed the effect of darkness, drought, and lack of nutrients in the substrate on the content and composition of phycobili proteins in terrestrial cyanobacterial strains. Total soluble proteins ranged from lowest of 0.29 mg/ml in P1 to highest of 0.70 mg/ml in P17 followed by strains P7 and P8 which showed almost similar total soluble proteins (0.65 mg/ml) (Table 1). Diversity in protein content was also shown amongst 20 cyanobacterial strains grown under different light and dark conditions (Prasanna *et al.* 2009).

Plant growth promoting attributes: The abundance of cyanobacteria in rice fields can be attributed to nitrogen fixing species which represent more than half of total

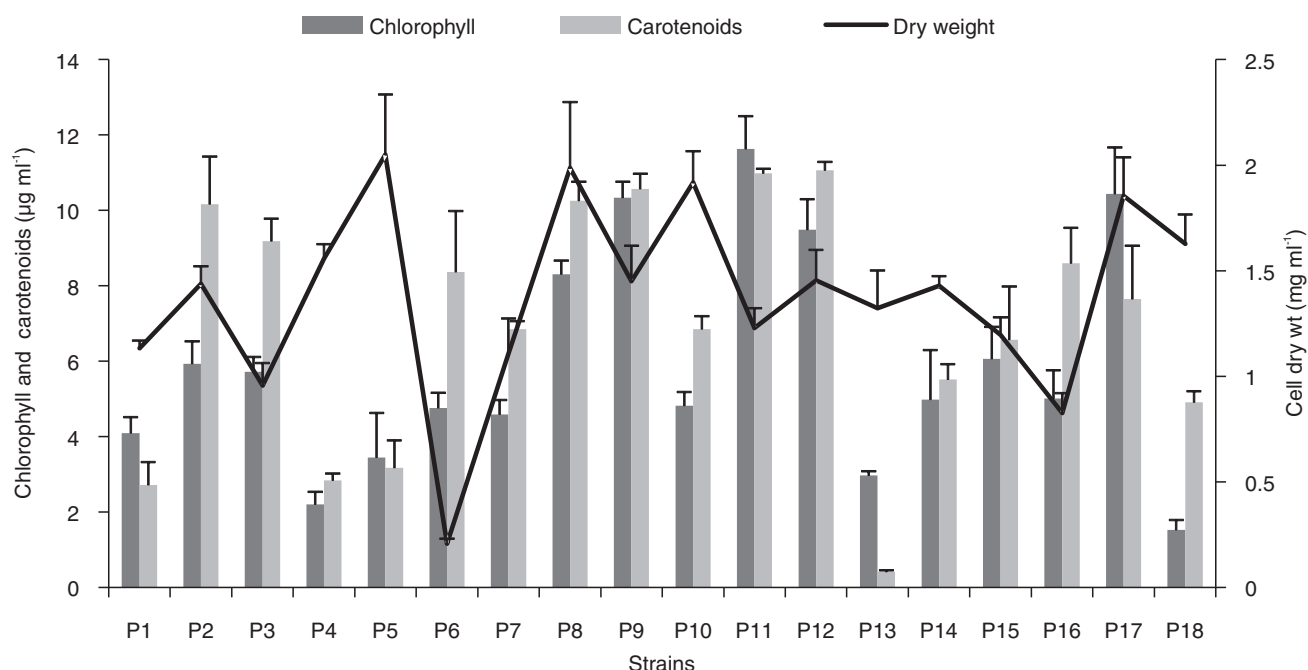


Fig 1 Comparative cell dry weight (mg/ml), chlorophyll ($\mu\text{g/ml}$) and carotenoids ($\mu\text{g/ml}$) amongst cyanobacterial strains from rice varieties of Punjab. Vertical bars on columns represent SD (n = 3).

Table 1 Comparative total soluble proteins (mg/ml), nitrogenase activity (nmole C_2H_4 mg/chl/ h), extracellular ammonia (μ mole NH_4^+ /ml) and indole acetic acid (μ g/ml) amongst cyanobacterial strains from rice varieties of Punjab

Designation	Total soluble proteins	Nitrogenase activity	Ammonia	Indole acetic acid
P1	0.29±0.015	1047.07±71.460	60.83±3.139	7.45±0.688
P2	0.40±0.020	772.16±69.745	31.70±2.551	43.54±3.192
P3	0.30±0.024	320.02±34.939	70.10±4.422	64.04±2.687
P4	0.35±0.005	746.02±47.683	55.00±2.364	9.54±1.422
P5	0.47±0.013	959.17±79.689	38.50±4.660	9.83±2.323
P6	0.35±0.021	458.54±45.401	52.70±2.406	39.29±1.078
P7	0.66±0.015	ND	72.00±1.216	61.54±1.855
P8	0.65±0.022	1049.41±55.407	19.90±2.325	13.62±1.965
P9	0.45±0.019	1109.44±17.357	105.16±2.709	15.87±2.291
P10	0.38±0.016	978.90±31.714	102.60±3.961	18.50±1.231
P11	0.33±0.027	736.21±31.164	229.70±6.720	22.58±1.063
P12	0.56±0.008	1180.65±52.144	69.07±0.892	42.16±0.402
P13	0.41±0.081	481.82±18.764	45.10±1.311	25.41±1.491
P14	0.33±0.008	738.01±37.951	107.46±2.223	9.87±0.901
P15	0.57±0.025	728.73±44.452	54.80±2.778	64.91±1.543
P16	0.40±0.014	610.56±34.580	33.66±2.478	18.33±1.377
P17	0.70±0.042	498.45±28.921	11.10±1.350	13.66±1.449
P18	0.45±0.014	ND	64.33±4.163	66.00±1.732
SE (±)	0.02	27.72	1.89	1.01
CD (P = 0.05)	0.04	80.05	5.42	2.90

Values after ± sign indicate standard deviation. Each value is a mean of three replications.

cyanobacteria which maintain fertility of rice field (De 1939). P7 and P18 did not exhibit nitrogenase activity under aerobic environment as these were non-heterocystous. Maximum nitrogenase activity of 1180.65 nmole C_2H_4 /mg chl/h was

observed in P12, an isolate of Parman-126, followed by P9 and P8 having activities of 1109.44 nmole C_2H_4 /mg chl/h and 1049.41 nmole C_2H_4 /mg chl/h. On the other hand, strain P3 of PB-1121 showed lowest activity of 320.02

nmole C_2H_4 /mg chl/h (Table 1). Based upon these observations, P12, P8, P9 can be studied further for their utility as an effective bio-inoculant to enhance nitrogen economy of rice field. Highest nitrogenase activity of 7.05 nmole/ml was reported in a study amongst 24 *Anabaena* strains (Nayak *et al.* 2007) while nitrogenase activity ranged from 602.40 nmole C_2H_4 /mg chl/h to 22939.70 nmole C_2H_4 /mg chl/h in a set of cyanobacterial strains (Prasanna *et al.* 2006).

It is necessary to select the cyanobacterial strains with non-repressible nitrogenase enzyme along with rapid growth and an ability to liberate significant ammonia to maximize crop yields (Rai and Prakasham 1991). Amongst 18 strains, extracellular ammonia release was

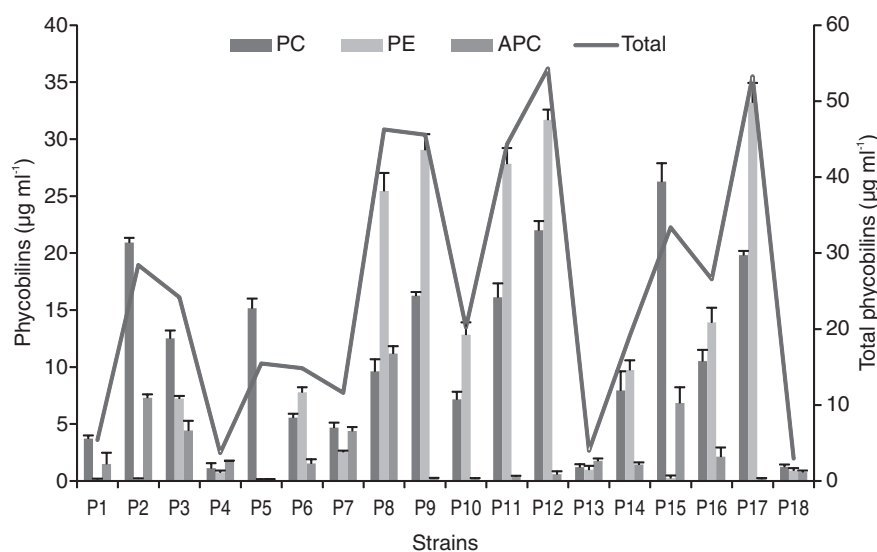


Fig 2 Comparative phycobilin proteins (μ g/ml) amongst cyanobacterial strains from rice varieties of Punjab. Phycocyanin (PC), Phycocerythrin (PE) and Allophycocyanin (APC). Vertical bars on columns represent SD (n = 3).

highest (229.70 $\mu\text{mole NH}_4^+/\text{ml}$) in P11, followed by P14 and P9 with ammonia release of 107.46 $\mu\text{mole NH}_4^+/\text{ml}$ and 105.16 $\mu\text{mole NH}_4^+/\text{ml}$, while, P17 recorded lowest extracellular ammonia of 11.10 $\mu\text{mole NH}_4^+/\text{ml}$ and all these belonged to rice variety Parman-126 (Table 1). In the present study, P11 can be a potential strain in region specific bioinoculant formulation as it exhibited highest ammonia excretion coupled with appreciable nitrogenase activity. Various factors such as chemical inhibitors, inorganic carbon, aeration rate, light intensity, light/dark cycle, shear stress, cell density, and culture depth alter ammonium excretion in cell free suspensions of heterocytous cyanobacteria (Dwivedi and Rai 1990). Ojit *et al.* (2016) studied the effect of different qualities (white, blue, green and red) of light on the ammonia excretion potential of six cyanobacterial strains and reported significant effect of these factors on extracellular ammonia release. Amongst strains tested, *Nostoc muscorum* BTA950 excreted maximum ammonia in white light followed by red light, green light and finally in blue light. On the other hand, ammonia excretion was negligible in *Anabaena*, with a maximum of 0.7 $\mu\text{mole NH}_4^+/\text{ml}$ (Prasanna *et al.* 2006).

The capability to secrete phytohormones is considered as a major property of rhizospheric, epiphytic and symbiotic microorganisms including cyanobacteria, besides members of the plant kingdom (Arshad and Frankenberger 1997). IAA production ranged from a lowest of 7.45 $\mu\text{g/ml}$ by P1 to highest of 66.00 $\mu\text{g/ml}$ by P18 (Table 1). Phytohormone (Indole-3-Acetic Acid) production potential by cyanobacteria has been reported by Sergeera *et al.* (2002). Release of IAA by several strains of *Anabaena* was observed in other studies as well (Prasanna *et al.* 2008b; Prasanna *et al.* 2009). Plant-associated microbe produced phytohormone has been implicated in associative or plant-microbe symbiotic interactions (Hirsch *et al.* 1997) in plant growth stimulation (Dobbelaere *et al.* 2003) and plant pathogenesis (Kado 1984). Diverse biological processes like cell division, elongation, differentiation, root elongation and tropistic responses are regulated by auxins (Spaepen *et al.* 2007). Based upon the results, cyanobacterial strains from rhizospheric soil or plant parts of rice varieties can be used for development of effective bioinoculant formulation for growth and nitrogen economy of rice crop.

The present study involved characterization of cyanobacterial strains isolated from rhizospheric soil and plant parts of selected rice varieties of Punjab, India. Results indicated the potential of specific strains for evaluation under pot culture and field trials based upon plant growth promoting attributes. Accordingly, these may have potential to be considered as efficient strains for the development of region specific bioinoculant formulations.

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