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Physiological Characterization and Electron Microscopic Investigation of Cyanobacteria Associated with Wheat Rhizosphere

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ABSTRACT. Physiological attributes of a set of cyanobacterial strains, isolated from the rhizosphere of wheat (var. HD 2687), identified as belonging to the genera *Calothrix* (*n* = 3), *Westiellopsis* (1), *Hapalosiphon* (2) and *Nostoc* (2), were axenized and evaluated. The concentrated culture filtrates of three cyanobacterial strains – *C. ghosei*, *H. intricatus* and *Nostoc* sp. were able to enhance germination percentage, radicle and coleoptile length in inhibition experiments with wheat seeds. Indole-3-acetic acid (IAA) production was recorded in light and dark (+0.5 % glucose) incubated cultures. Incubation in the presence of tryptophan significantly enhanced IAA production. Acetylene-reducing activity was higher in light incubated cultures of *Nostoc* sp. followed by *C. ghosei*, while in the dark, *C. ghosei* recorded highest values. TLC of the filtrates revealed the presence of several amino acids such as histidine, and auxin-like compounds. Co-culturing with selected strains recorded significant enhancement in plant chlorophyll. Root sections of wheat seedlings co-cultured with *C. ghosei* revealed the presence of short filaments inside the root hairs and cortical region. Such strains can be promising candidates for developing plant growth promoting associations for wheat crop, besides serving as model systems for understanding the metabolic interactions of cyanobacteria with host plant, such as wheat.

Abbreviations

ARA	acetylene-reducing activity
BGA	blue-green algae
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MPN	most probable number
TLC	thin layer chromatography
C.	*Calothrix*
H.	*Hapalosiphon*
N.	*Nostoc*
W.	*Westiellopsis*

The significance of these phototropic prokaryotes in agriculture was first recognized by De (1939) who attributed the self-maintenance of the N-structure of tropical rice field soils to the growth of N_2-fixing BGA, which also bring about a yield improvement of 5–25 % in rice, even in the presence of high doses of nitrogenous fertilizers (Venkataraman 1981; Yanni *et al*. 1992). Many of them have the capacity to form specific associations with protist animals and plants, ranging from unicellular algae to angiosperms, including fungi (Rai *et al*. 2000). Such mutualistic processes are known to be mediated through signaling mechanisms, which are currently of major interest to researchers (Rai *et al*. 2000; Rai and Bergman 2002). The nitrogen fixed by the cyanobiont is used by the fungal or plant partner, which in turn, supplies the nutritional substances and a niche for the growth and survival of the cyanobiont (Rai 1990). At present, scientists are interested in creating artificial symbiosis between higher plants and N_2-fixing microorganisms with a view to introducing nitrogen-fixing ability in the plants and develop beneficial mutualistic associations. In this context, Gantar *et al.* (1991*a,b*) observed intriguing associations of selected cyanobacterial strains with wheat seedlings.

The rhizosphere is well recognized as the hot spot for microbial abundance and diversity, and metabolic activities intimately related to the successful production of crops and sustenance of soil fertility. However, the rhizosphere of crop plants is a relatively unexplored frontier in terms of cyanobacterial abundance, diversity and the general belief that cyanobacteria are obligate phototrophs has been perhaps the major reason for the dearth of information on these organisms in this niche. Most of the published work on BGA biofertilizers has been carried out in relation to rice crop, with few reports on their effects on vegetables, wheat, or other horticultural crops (Venkataraman 1981). Wheat is an agriculturally significant crop, in which high doses of fertilizers are required for enhancing crop yields. However, biofertilizers such as BGA have not

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been exploited as supplementary inputs in wheat and very scanty information is available on the association of cyanobacteria with this crop (Prasanna *et al.* 2008).

Therefore, our aim was to evaluate the role of indigenous cyanobacteria isolated from the rhizosphere of wheat plants, with major emphasis on the production of growth promoting substances, as a prelude to developing plant growth promoting associations for this crop.

MATERIALS AND METHODS

Enrichment, isolation and purification of cyanobacterial strains from wheat rhizosphere. Rhizospheric soil samples were collected from experimental fields of wheat crop (cv. HD2687) belonging to our institute. The soil samples (5 g portions) were transferred to 100 mL of sterilized N free BG-11 medium. The flasks were shaken well and incubated undisturbed for 21 d at 30 ± 2 °C and 2–3 klx light intensity (16 : 8 h, L : D) under controlled conditions of illumination and temperature. Enumeration of cyanobacteria in the rhizosphere was done by the MPN method (Prasanna *et al*. 2006). The cyanobacterial abundance in the samples was expressed as CFU/g of soil. The enrichment flasks and MPN tubes were regularly monitored for growth and observed microscopically. Standard plating/streaking techniques were used for isolation and purification of cyanobacterial strains (Stanier *et al*. 1971).

The cyanobacterial strains were made axenic through repeated antibiotic treatments in the dark (using a mixture of streptomycin sulfate, chloramphenicol and tetracycline at standard doses) followed by testing for the absence of turbidity after incubation in nutrient broth (Kaushik 1987). Batch cultures of such axenized strains were grown up to exponential phase (15 d) under optimum environmental conditions, after uniform inoculation at the rate of 5 % cyanobacterial suspension. The cultures were maintained in chemically defined nitrogen-free BG-11 medium (Stanier *et al.* 1971) at 27 ± 2 °C under a light intensity of 52–55 μmol photon per m^2 per s (16 : 8 h; L : D). Such cultures were homogenized (to break the clumps and obtain a uniform suspension) and triplicate samples from independent flasks were used for analyses of morphological, physiological and biochemical attributes.

Morphological characterization and identification. The cyanobacterial strains were examined for their morphological characteristics in liquid and agar-based medium. The color of thallus, planktonic/benthic nature and type of growth were examined. The microscopic measurements of cell shape, size/observations on each strain were compared with the keys given by Desikachary (1959) and tentative identification up to the species level was done.

Physiological characterization. Homogenized suspensions of late exponential-phase cultures were utilized for the biochemical and physiological analyses. Extraction of proteins was done from 0.5 mL of cyanobacterial suspension by the addition of 0.5 mL of 1 mol/L NaOH and placing the samples in boiling water bath for 10 min. After extraction, the methodology of Herbert *et al.* (1977) was followed and the absorbance was read at 650 nm. The amount of protein was calculated from the standard curve of known amounts of bovine serum albumin and expressed as μg of protein per mL of culture.

Effect of cyanobacterial culture filtrates on germination of wheat seeds. Late exponential-phase cultures of cyanobacteria were centrifuged at 60 Hz for 5 min and the pellet and supernatant were collected separately. The supernatant was lyophilized using Christ L-1, Alpha 2-41 model, at –45 °C, resuspended in 10 % of the original volume of sterile water and used to assess the influence of extracellular substances on wheat seeds/seedlings.

In germination experiments, selected cyanobacterial filtrates were bioassayed for their ability to promote or inhibit seedling growth using method of Shende *et al*. (1977) with a few modifications. Growth tests involved three replications of 20 seeds of wheat variety HD 2687 for each strain. The seeds were surface-sterilized in 70 % ethanol for 30 s and then in 0.1 % $HgCl_2$ solution for 5 min. Finally, they were rinsed several times with sterile water before use. Surface-sterilized seeds were soaked in the concentrated culture filtrates for 3 h under aseptic conditions and placed in seedling agar plates (containing 2 % agar) for germinating the seeds. In control plates, seeds were placed after soaking in sterile water for 3 h. The parameters (percent germination and length of radicle or coleoptile) were employed to assess the influence of cyanobacterial filtrates on the germination and growth of wheat seedlings. Germination was recorded after 1, 2, and 3 d. The entire experiment was conducted in a growth chamber set at 27 °C.

Gas chromatographic quantification of ethylene formed by the suspension was utilized as an index of nitrogenase activity and expressed as ARA, vials with equivalent volume of water serving as controls (Prasanna *et al*. 2002). Fifteen-mL glass vials with 5 mL cyanobacterial cultures were incubated under a gas mixture which had been substituted with 10 % acetylene under standard growth conditions for 1½ h. One-mL aliquots of gaseous phase were removed and injected into preconditioned *Nucon* model GC 5500 housing

a 2-m long Porapak R stainless steel column and a flame ionization detector. The column temperature was maintained at 100 °C, injector and detector temperature at 110 °C. A flow rate of 35 mL/min of N_2 was maintained for the carrier gas. Standard ethylene–argon mixture was used for calibration. The ARA values are means of triplicate measurements and are expressed per μg protein.

BG-11 medium was amended with glucose at 5 g/L and Trp at 500 mg/L (as precursor of IAA) for evaluating the potential of these strains to produce IAA in the dark. Trp stock solution was prepared in 50 % ethanol. Both Trp and dextrose solution were filter sterilized. The cyanobacterial inoculum was added at 5 % of the medium volume. The tubes were incubated in growth room in the dark (with glucose) and light (without glucose), respectively, in the presence and absence of Trp. The filtrate was checked for the production of IAA after four weeks of incubation, following the method of Gordon and Weber (1951) and growth was evaluated in terms of protein content.

Co-culturing of wheat seedlings with cyanobacterial strains. The selected cyanobacterial isolates were co-cultured with wheat seedlings, placed in a sterilized setup consisting of 50 mL test tubes containing 20 mL of N-free BG-11 medium and 10 mL plastic pipette tip cut to facilitate the placing of seedling. Three-d-old seedlings of wheat variety HD 2687, obtained from surface-sterilized seeds grown on seedling agar, were placed such that the root tip was in contact with the medium. Cyanobacterial inoculum was added at 10 % of the medium volume. Three replications (each consisting of three seedlings) were assayed. In control tubes, no inoculum was added, and a set of controls with only cyanobacterial inoculum was also included. The lower portion of the tubes was covered with aluminum foil to provide conditions simulating soil. The entire setup was kept in a growth chamber, maintained at 27 ± 2 °C and illumination of 2.5–3 klx light intensity (12 :12 h; L : D). After 10 d, plant growth parameters were recorded, including fresh mass. Plant chlorophyll was estimated spectrophotometrically (Jeffrey and Humphrey 1975) after excising the roots. Thin sections of roots (transverse and longitudinal) were prepared by microtomy and fixed (Brandon *et al.* 1964), and observed under a light microscope to study the cyanobacterial association with the plant roots, followed by transmission electron microscopy at the Sophisticated Analytical Instrumentation Facility (*All India Institute of Medical Sciences*, New Delhi, India). Root sections were prepared after fixation in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4 °C and embedding in LR-White resin.

The methodology of Pillay and Mehdi (1968) was followed for the identification of auxins by TLC. Samples (20 μL) of cyanobacterial culture filtrate were spotted on TLC plates, along with 5 μL of standard solutions (1 mg/mL in 95 % ethanol) of IAA and IBA. The plates were developed with 2-propanol–water (3 : 1, *V*/*V*) and sprayed with Van Urk's reagent, and R_f values were compared with standards.

The methodology of Kerkut and Shapira (1968) involving the use of TLC was followed for the identification of free amino acids. Five μL of standard amino acid solutions (1 % standard amino acid solutions in 10 %, *V*/*V*, 2-propanol) were spotted on the pre-activated silica plates along with samples (20 μL). The chromatogram was developed in 1-butanol–acetic acid–water (3 :1 :1, *V*/*V*), sprayed with ninhydrin and heated for 10 min at 110 °C. The R_f values were compared with those of the amino acid standards. The culture filtrate and extract (before and after concentration) were scanned in the UV-VIS range using spectrophotometer (*Specord*), along with IAA and IBA standards.

Statistical analyses. The triplicate sets of data for the various parameters evaluated were subjected to ANOVA in accordance with the experimental design (completely randomized design) using MSTAT-C statistical package to quantify and evaluate the source of variation, and critical difference values were calculated at p level of 0.05 %.

RESULTS AND DISCUSSION

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 (Rai 1990; Rai *et al*. 2000; Sood *et al.* 2008; Zapomělová *et al.* 2008). However, the below-ground diversity of these organisms has been less investigated, especially in crop plants.

We found that the population of nitrogen fixing cyanobacteria in the rhizosphere of wheat plants (variety HD 2687) was 2.6×10^2 CFU/g soil, while non-nitrogen-fixing cyanobacterial population was 1.47×10^5 CFU/g soil. Eight cyanobacterial isolates belonging to eight different heterocystous genera (identified as *C. ghosei*, *H. intricatus*, *Nostoc* sp., *Hapalosiphon* sp., *Calothrix* sp., *N. muscorum*, *W. prolifica*, *C. membranacea*) based on taxonomic keys of Desikachary (1959), were obtained from the rhizosphere soil samples of wheat. After axenization using triple antibiotic treatment, these strains were characterized in terms of their morphological and physiological attributes.

The production of phytohormones (earlier considered as a trait of the plant kingdom) is also widespread among soil and plant associated prokaryotes, especially those involved in associative interactions with plant or pathogenesis (Costacurta and Vanderleyden 1995). Plant growth promoting rhizobacteria are known to enhance crop yields by several mechanisms, among which foremost is the synthesis of phytohormones, which play an important role as signals and regulators of growth and development (Glick *et al*. 1999). Genetic mechanisms underlying IAA biosynthesis and its regulation have been studied in a number of microorganisms, including *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Agrobacterium*, *Nostoc* sp. (Rasmussen *et al*. 1994; Sergeeva *et al*. 2002). The growth promoting effect of cyanobacteria on wheat crop is a less investigated area of research, as cyanobacterial biofertilizers do not form part of the integrated nutrient management practices for this crop.

Soaking of seeds of wheat variety HD2687 under controlled conditions in the different cyanobacterial culture filtrates did not result in significant differences in germination percentage of seeds (Table I). The overall germination percentage varied in the range of 96–99 %. On the other hand, when the length of coleoptile was measured in the seedlings after 3 d, highly significant values were recorded in the treatment involving soaking in extracellular filtrates of strain *Nostoc* sp. followed by *C. ghosei*. These isolates brought about 50–60 % enhancement in the length of radicle and coleoptile and 7–9 % increase in seed germination.

Table I. Effect of extracellular filtrates of rhizocyanobacteria on the germination of wheat seeds after 3 d

Treatment	Length of coleoptile mm	Length of radicle mm	Germination %
Sterile water	7.7	27.3	90
Sterile BG-11 medium	10.3	34.2	90
Calothrix ghosei	19.8	58.0	99
Hapalosiphon intricatus	17.1	51.7	99
Nostoc sp.	20.9	48.3	99
Hapalosiphon sp.	13.3	42.9	98
Calothrix sp.	14.3	41.5	98
Nostoc muscorum	14.5	43.1	97
Westiellopsis prolifica	14.7	44.7	98
Calothrix membranacea	11.3	45.0	98
CD[a] ($p = 0.05$)	1.7	2.7	–
SEd[b]	0.80	1.3	–

[a]Critical difference. [b]Standard error (deviation).

Physiological characterization of the rhizo-cyanobacteria was done in terms of IAA production, total soluble proteins and ARA. We observed that IAA production proceeded both under light and dark (+0.5 % glucose) in all the cyanobacteria evaluated (Table II) and ranged from 0.6 (*C. membranacea*) to 3.37 μg/mL in the culture filtrates of light-grown cultures, and from 0.5 (*Hapalosiphon* sp.) to 1.39 (*Nostoc* sp.) μg/mL in cultures incubated in the dark, in the presence of 0.5 % glucose. *C. ghosei*, *H. intricatus* and *Nostoc* sp. produced the highest amount of IAA under both conditions. The extracellular filtrates of the cyanobacterial strains were scanned in the range of 200–300 nm. Distinct peaks were observed at 296 and 300 nm (λ_{max} for the standard IAA and IBA, respectively). The ability of cyanobacteria to produce the phytohormone IAA was confirmed by colorimetric (Salkowski) method. Sergeeva *et al.* (2002) using similar assays observed that auxin-like compounds were released by ≈38 % of the free living as compared to 83 % of the symbiotic isolates and confirmed the constitutive or Trp dependent production of IAA, by ELISA test

Table II. Physiological characterization of the selected rhizocyanobacterial strains grown under light and dark (+0.5 % glucose)

Isolate	ARA, nmol[a]		IAA[b], μg/mL		Protein[c], μg/mL	
	light	dark	light	dark	light	dark
Calothrix ghosei	655	41.4	3.37	0.93	218	30.4
Hapalosiphon intricatus	597	21.1	2.70	1.39	123	34.5
Nostoc sp.	689	28.7	2.19	0.76	88.7	32.6
Hapalosiphon sp.	156	9.31	1.19	0.50	112	24.5
Calothrix sp.	389	7.16	1.86	1.00	191	26.5
Nostoc muscorum	513	18.3	2.09	0.62	122	11.4
Westiellopsis prolifica	345	15.8	1.16	0.17	149	27.5
Calothrix membranacea	125	12.5	0.60	0.54	192	20.8
CD ($p = 0.05$)	9.30	1.43	0.21	0.13	5.91	1.23
SEd	4.39	0.67	0.10	0.06	2.79	0.58

[a]Per μg protein per h. [b]In culture filtrate. [c]Total soluble.

and putative presence of *ipdC* gene homologues. Venkataraman and Neelakantan (1967) had shown that biologically active cell constituents such as amino acids, auxins and vitamin B_{12} are involved in the fertilizing action of BGA. Misra and Kaushik (1989*a*,*b*) also analyzed cellular and extracellular growth promoting substances liberated by *N. muscorum* and *H. fontinalis*, which included vitamins, IAA, amino acids and sugars.

Among the several pathways and intermediates involved in generating IAA, Trp is considered as the main precursor. Comparative analysis of IAA production in the three promising strains (*C. ghosei*, *H. intricatus*, *Nostoc* sp.) in the presence and/or absence of Trp revealed interesting results, especially in the dark-incubated cultures (Table III). *C. ghosei* exhibited a 2- and 9-fold enhancement in IAA production under light and dark when Trp was added, and recorded highest values of IAA (9.33 μg/mL) in dark-incubated cultures amended with Trp (Fig. 1). IAA production was also evaluated by TLC, which indicated seven strains showing positive spots with R_f similar to pure IAA; filtrates were measured for all the cultures. Our data indicate that these strains may be able to convert exogenous Trp into IAA or use Trp as a source of nitrogen during the initial stages of incubation. Ahmad *et al*. (2005) observed that in the presence of Trp, *Azotobacter* and *Pseudomonas* produced 7.3–32.8 μg/mL IAA compared with 2.68–10.8 in the absence of Trp. *Pseudomonas* produced 41–53.2 μg/mL in the presence of Trp as compared with 5.34–9.64 μg/mL IAA in the absence of Trp. Use of labeled Trp and its sensitive measurement using GC–MS may aid in determining the metabolic pathway and intermediates involved in its utilization by cyanobacteria.

Table III. Effect of tryptophan on IAA production (μg/mL) by cyanobacteria in light and dark regime after incubation for 14 d

Isolate	Light	Dark
Calothrix ghosei	5.66	9.33
Hapalosiphon intricatus	3.58	4.56
Nostoc sp.	3.67	3.76
Hapalosiphon sp.	1.54	3.40
Calothrix sp.	3.11	3.18
Nostoc muscorum	1.34	3.52
Westiellopsis prolifica	1.26	1.97
Calothrix membranacea	2.52	2.48
CD ($p = 0.05$)	0.16	0.55
SEd	0.08	0.26

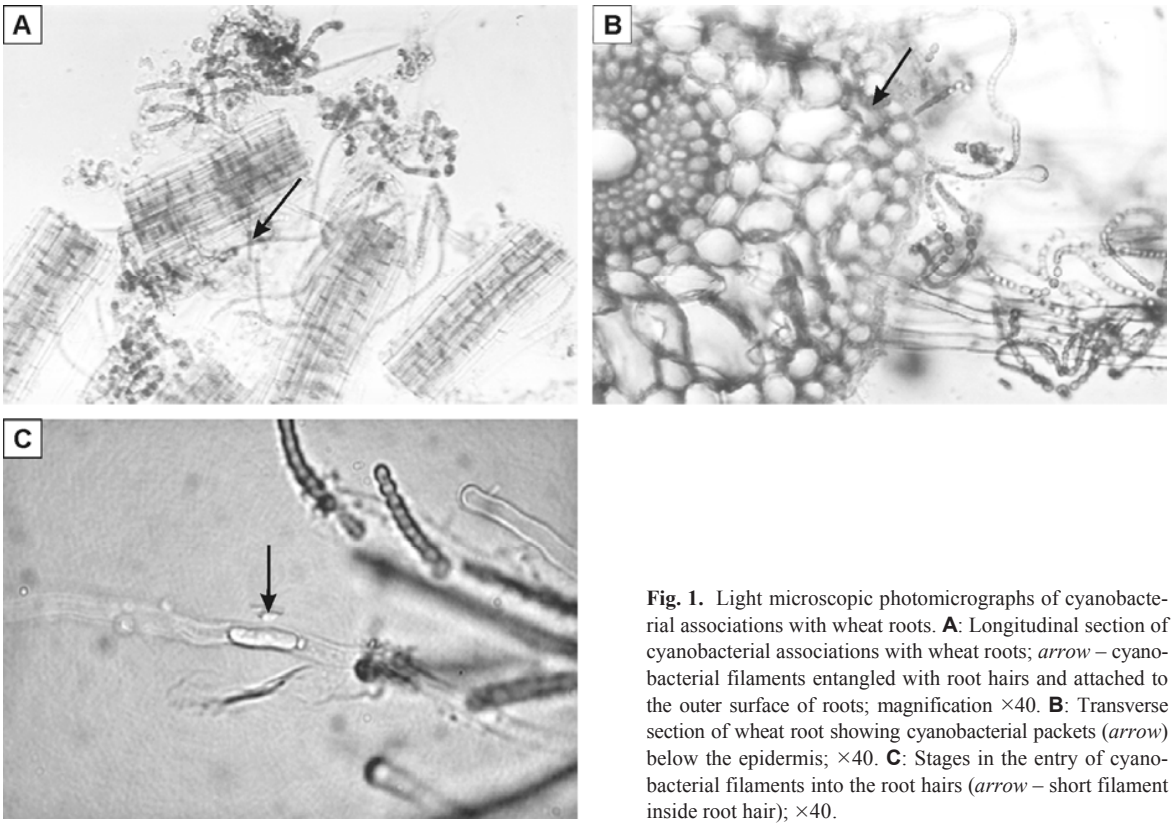

Fig. 1. Light microscopic photomicrographs of cyanobacterial associations with wheat roots. **A**: Longitudinal section of cyanobacterial associations with wheat roots; *arrow* – cyanobacterial filaments entangled with root hairs and attached to the outer surface of roots; magnification ×40. **B**: Transverse section of wheat root showing cyanobacterial packets (*arrow*) below the epidermis; ×40. **C**: Stages in the entry of cyanobacterial filaments into the root hairs (*arrow* – short filament inside root hair); ×40.

The production of combined nitrogen in soluble form by nitrogen fixing cyanobacteria has been recorded by De (1939) and Fogg (1952). TLC of the filtrates of cultures grown in the light showed the presence of an array of amino acids (Table IV). The R_f values of the separated components of the filtrates of the cyanobacterial strains were compared with those of standard amino acids. Histidine was the prominent

amino acid in the culture filtrates of all strains, except *N. muscorum*. The culture filtrates of *N. muscorum*, *H. intricatus* and *Nostoc* sp. revealed the presence of glycine, which along with aspartic acid and arginine formed the resolved spots for culture filtrates of *W. prolifica* and *C. membranacea.* The culture filtrate of *N. muscorum* contained cystine and leucine. Extracellular amino acids have been reported in a number of cyanobacterial strains such as *C. brevissima*, *Anabaena cylindrica* and a set of unicellular and heterocystous forms (Fogg 1952). Among the various amino acids, aspartate, glutamate and alanine have been reported in *Anabaena azollae* and *Nostoc* sp. (Venkataraman and Saxena 1963). Singh and Trehan (1973) reported the predominance of aspartate in the extracellular filtrates of *Aulosira fertilissima* and *Anacystis nidulans*, along with proline, valine, and glycine at various stages of growth. Evaluation of ARA also revealed that its activity was observed in *Nostoc* sp. followed by *C. ghosei* and *H. intricatus* (Table II). In the dark, the ARA activity was the highest in *C. ghosei* and lowest in *Calothrix* sp. A 10–20× decrease was observed as compared to light-incubated cultures. The liberation of extracellular nitrogenous substances, mostly fixed through nitrogenase→glutamate–ammonia ligase ('glutamine synthetase') →glutamate synthase pathway by cyanobacteria is known to have wide ranging implications in the immediate growth environment, leading to proliferation of microflora, which can use these amino acids.

Table IV. Chemical analysis of extracellular filtrates of rhizocyanobacteria

Strain	Extracellular protein µg/mL	Amino acids detected
Calothrix ghosei	82.3	His, Gly
Hapalosiphon intricatus	80.0	His, Gly
Nostoc sp.	62.7	His, Gly
Hapalosiphon sp.	41.8	His, Leu, Asp
Calothrix sp.	45.0	His, Pro/Hyp[a], Gly
Nostoc muscorum	39.3	Cys, Leu
Westiellopsis prolifica	25.3	His, Gly, Asp
Calothrix membranacea	32.3	His, Gly, Arg
CD ($p = 0.05$)	3.09	
SEd	1.80	

[a]4-Hydroxyproline.

A significant enhancement in plant chlorophyll was recorded over control (wheat seedlings grown in sterile medium) in the co-culturing experiment conducted with the three promising strains using wheat seedlings. Highest values were recorded on co-culturing with *Nostoc* sp. (K3) strain followed by *C. ghosei* (K1) and *H. intricatus* (K2) (Fig. 2). ARA was highest in wheat seedlings co-cultured with *Nostoc* sp. (K3)

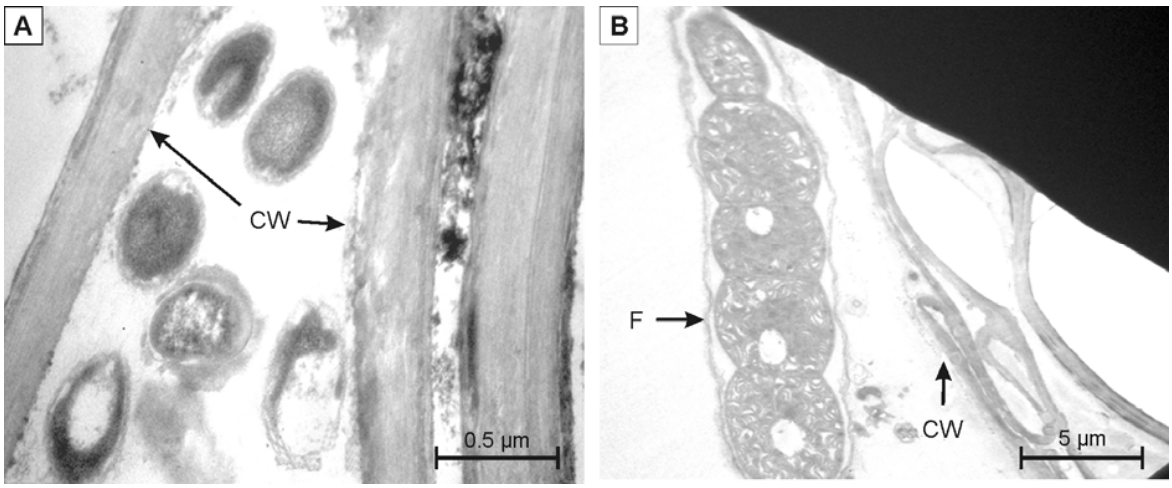

Fig. 2. Transmission electron micrographs of wheat root sections showing the intracellular location of single cells and short filaments of *C. ghosei* within the host cells; CW – cell wall of the host plant, F – cyanobacterial filament.

followed by *C. ghosei* (K1). Control seedlings did not show any ARA. Wheat seedlings + *H. intricatus* (K2) exhibited the lowest ARA activity of 0.359 nmol/g fresh biomass per h (Table V).

Microscopic analyses of wheat roots co-cultured with *C. ghosei* revealed interesting observations. Transmission electron microscopy (Fig. 3) of the root sections clearly revealed that *Calothrix* sp. penetrated the cortical region through root hairs. In symbiotic tissues of plant cyanobacterial associations, the filamentous habit of the cyanobionts tends to become aseriate and sometimes unicellular or two-celled structures may be observed (Rai 1990; Rai *et al.* 2000). Our *Calothrix* filaments were much shorter, with thin walls.

The extension of the range of plants with the ability to fix nitrogen has been a long-term objective of plant scientists, which can have great environmental and economic significance. In this context, cyanobacteria are particularly suitable for the creation of novel nitrogen-fixing associations since they already form a wide variety of natural symbiotic associations (Gusev *et al*. 1986; Rai 1990; Rai *et al.* 2000). Nilsson *et al*. (2002, 2005, 2006) tested numerous symbiotic cyanobacterial isolates for their ability to associate with rice and demonstrated that symbiotically competent *Nostoc* strains respond chemotactically to extracts of natural host and non-host plants. Gantar *et al.* (1991*a*,*b*) demonstrated the ability of certain soil isolates of cyanobacteria of the genera *Nostoc* and *Anabaena* to form intimate associations with the roots of wheat plants; cyanobacteria were observed to exist as hormogonia of heterocystous filaments or aseriate packages in intercellular spaces or occasionally filaments were observed inside empty looking root cells.

Table V. Co-culturing of selected cyanobacterial strains with wheat (var. HD 2687) in hydroponics and their effect on fresh biomass and ARA

Treatment[a]	Fresh biomass mg	ARA nmol[b]
+ *Calothrix ghosei*	265	1.09
+ *Hapalosiphon intricatus*	214	0.359
+ *Nostoc* sp.	200	1.77
+ Sterile medium	153	–
CD ($p = 0.05$)	20	0.06
SEd	10	0.03

[a]Wheat seedlings. [b]Per g fresh biomass per h.

Short filaments of our *Calothrix* were clearly visible in the root hair cells, perhaps the site of entry into the root tissues. Further, these cells or short filaments penetrated the epidermal and cortical regions, and were also observed in intercellular space, indicating a tight association. West and Adams (1997) reported that besides *Nostoc*, *Calothrix* and *Chlorogloeopsis* also form association with *Anthoceros* or *Blasia*. Chemotaxis is known to be an important factor influencing cyanobacterial associations with higher plants. Symbiotically competent *Nostoc* strains are known

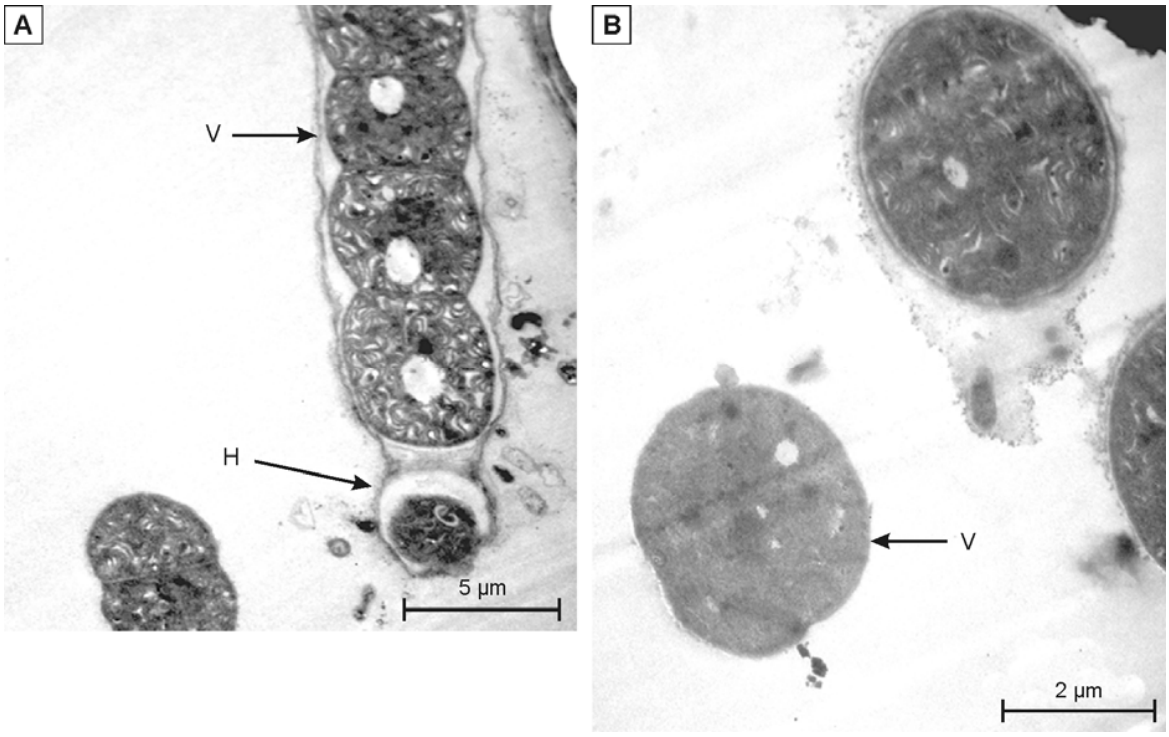

Fig. 3. Transmission electron micrographs of wheat root sections showing the intracellular location of single cells and short filaments of *C. ghosei* with heterocyst (H) and vegetative (V) cells.

to respond chemotactically to extracts or exudates of hosts or non-host plants. Nilsson *et al*. (2006) suggested the widespread presence of chemo-attracting substances in plants. According to us, both auxins (IAA, IBA) and amino acids produced by cyanobacteria may definitely form part of the signaling cascade existing in the host–cyanobiont interactions occurring in the wheat rhizosphere.

The plant growth promoting activity of these strains has already been established in pot experiments under glasshouse and controlled conditions (Karthikeyan *et al*. 2007). Therefore, future research needs to be done for field level testing of the three cyanobacterial strains identified by us which show potential as inoculants, due to the extracellular production of IAA and amino acids and high nitrogenase activity in light and dark environments.

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REFERENCES

AHAMAD F., AHAMAD I., KHAN M.S.: Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Turk.J.Biol*. **29**, 29–34 (2005).

COSTACURTA A., VANDERLEYDEN J.: Synthesis of phytohormones by plant-associated bacteria. *Crit.Rev.Microbiol*. **21**, 1–18 (1995).

DE P.K.: The role of blue green algae in nitrogen fixation in rice fields. *Proc.Roy.Soc.London Ser*. **B 12,** 129–139 (1939).

DESIKACHARY T.V.: *Cyanophyta* (ICAR Monographs on algae). Indian Council of Agricultural Research, New Delhi 1959.

FOGG G.E.: The production of extracellular nitrogenous substances by a blue green alga. *Proc.Roy.Soc.London* **139B**, 372–397 (1952).

GANTAR M., KERBY N.W., ROWELL P., OBREHT Z.: Colonization of wheat (*Triticum vulgare* L.) by nitrogen fixing cyanobacteria. I. A survey of soil cyanobacterial isolates forming associations with roots. *New Phytol*. **118**, 477–483 (1991*a*).

GANTAR M., KERBY N.W., ROWELL P.: Colonization of wheat (*Triticum vulgare* L.) by N_2 fixing cyanobacteria – II. An ultrastructural study. *New Phytol.* **118**, 485–492 (1991*b*).

GLICK B.R., PATTEN C.L., HOLGUIM G., PENROSE D.M.: *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria*. ICP, Covent Garden, London 1999.

GORDON A.S., WEBER R.P.: Colorimetric estimation of indole acetic acid. *Plant Physiol*. **26**, 192–195 (1951).

GUSEV M.V., KORZHENEVSKAYA T.G., PYVOVAROVA L.V., BAULINA O.I., BUTENKO R.G.: Introduction of nitrogen-fixing cyanobacterium into shoot regenerates. *Planta* **167**, 1–8 (1986).

HERBERT D., PHIPPS P.J., STRANGE R.E.: Chemical analysis of microbial cells, pp. 209–304 in *Methods in Microbiology*, Vol. 5B. Academic Press, New York 1971.

JEFFREY S.W., HUMPHREY G.F.: New spectrophotometric equations determining chl*a*, *b*, *c* and c_2 in higher plants, algae and natural phytoplankton. *Biochem.Biophysiol.Pfl*. **167**, 191–194 (1975).

KARTHIKEYAN N., PRASANNA R., LATA, KAUSHIK B.D.: Evaluating the potential of plant growth promoting cyanobacteria as inoculants for wheat. *Eur.J.Soil Biol.* **43**, 23–30 (2007).

KAUSHIK B.D.: *Laboratory Methods for Blue-Green Algae*. Assoc. Publ. Co, New Delhi 1987.

KAUSHIK B.D., VENKATARAMAN G.S.: Response of cyanobacterial nitrogen fixation to insecticides. *Curr.Sci*. **52**, 321–323 (1983).

KERKUT G.A., SHAPIRA A.: *Experiments in Physiology and Biochemistry*, pp. 352–359 in G.A. Kerkut (Ed.). Academic Press, London 1968.

KNIGHT C.D., ADAMS D.G.: A method for studying chemotaxis in nitrogen-fixing cyanobacterium–plant symbioses. *Physiol.Mol.Plant Pathol*. **9**, 73–77 (1996).

MISRA S., KAUSHIK B.D.: Growth promoting substances of cyanobacteria. I. Vitamins and their influence on rice plant. *Proc.Indian Sci.Acad.* **B55**, 295–300 (1989*a*).

MISRA S., KAUSHIK B.D.: Growth promoting substances of cyanobacteria. II. Detection of amino acids, sugars and auxins. *Proc.Indian Sci.Acad*. **B55**, 499–504 (1989*b*).

NILSSON M., BHATTACHARYA J., RAI A.N., BERGMAN B.: Colonization of root of rice (*Oryza sativa*) by symbiotic *Nostoc* strains. *New Phytol*. **156**, 517–525 (2002).

NILSSON M., RASMUSSEN U., BERGMAN B.: Cyanobacterial chemotaxis to extracts of host and non-host plants. *FEMS Microbiol.Ecol*. **55**, 382–390 (2006).

NILSSON M., RASMUSSEN U., BERGMAN B.: Competition among symbiotic cyanobacterial *Nostoc* strains forming artificial associations with rice (*Oryza sativa*). *FEMS Microbiol.Lett*. **245**, 139–144 (2005).

PILLAY D.N., MEHDI R.: Separation of simple indole derivatives by thin layer chromatography. *J.Chromatogr.* **32**, 592 (1968).

PRASANNA R., MOHAMMADI S.A., PRASANNA B.M., SINGH P.K.: Inter- and intra-specific variation for nitrogen-fixing potential in *Tolypothrix* germplasm. *Acta Bot.Hungar*. **44**, 383–392 (2002).

PRASANNA R., SAXENA A.K., JAISWAL P., NAYAK S.: Development of alternative support system for viable count of cyanobacteria by MPN method. *Folia Microbiol*. **51**, 455–458 (2006).

Prasanna R., Jaiswal P. Singh Y.V., Singh P.K.: Influence of biofertilizers and organic amendments on nitrogenase activity and phototrophic biomass of soil under wheat. *Acta Agron.Hungarica* 56, 149–159 (2008).

RAI A.N.: *Handbook of Symbiotic Cyanobacteria*. CRC Press, Boca Raton, FL (USA) 1990.

RAI A.N., BERGMAN B.: Creation of new nitrogen-fixing cyanobacteria associations. *Proc.Roy.Irish Acad. (Supl. Issue – Biology and Environment)* **102B**, 65–68 (2002).

RAI A.N., SÖDERBÄCK E., BERGMAN B.: Cyanobacterium–plant symbioses. *New Phytol.* **147**, 449–481 (2000).

RASMUSSEN U., JOHANSSON C., BERGMAN B.: Early communication in the *Gunnera–Nostoc* symbiosis: plant-induced cell differentiation and protein synthesis in the cyanobacterium. *Mol.Plant Microbe Inter.* **7**, 696–702 (1994).

SERGEEVA E., LIAIMER A., BERGMAN B.: Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. *Planta* **215**, 229–238 (2002).

SHENDE S.T., APTE R.G., SINGH T.: Influence of *Azotobacter* on germination of rice and cotton seeds. *Curr.Sci.* **46**, 675–676 (1977).

SINGH V.P., TREHAN T.: Effect of extracellular products of *Aulosira fertilissima* on the growth of rice seedlings. *Plant Soil* **38**, 457–464 (1973).

SOOD A., PRASANNA R., PRASANNA B.M., SINGH P.K.: Genetic diversity among and within cultured cyanobionts of diverse species of *Azolla. Folia Microbiol.* **53**, 35–43 (2008).

STANIER R.Y., KUNISAWA R., MANDEL M., COHEN-BAZIRE G.: Purification and properties of unicellular blue green algae (Order: *Chroococcales*). *Bacteriol.Rev.* **35**, 171–305 (1971).

VENKATARAMAN G.S.: *Blue-Green Algae for Rice Production – a Manual for Its Promotion*. FAO Soils Bull. no. 46. FAO, Rome 1981.

VENKATARAMAN G.S., NEELAKANTAN S.: Effect of cellular constituents of nitrogen fixing blue-green alga *Cylindrospermum* on root growth of rice plants. *J.Gen.Appl.Microbiol*. **13**, 53–62 (1967).

VENKATARAMAN G.S., SAXENA H.K.: Studies on nitrogen fixation by blue green algae. IV. Liberation of free amino acids in the medium. *Indian J.Agric.Sci.* **33**, 22–24 (1963).

WEST N.J., ADAMS D.G.: Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site. *Appl.Environ.Microbiol.* **63**, 4479–4484 (1997).

YANNI Y.G.: The effect of cyanobacteria and *Azolla* on the performance of rice under different levels of fertilizer nitrogen. *World J.Microbiol.Biotechnol.* **8**, 132–136 (1992).

ZAPOMĚLOVÁ E., HROUZEK P., ŘEHÁKOVÁ K., ŠABACKÁ M., STIBAL M., CAISOVÁ L., KOMÁRKOVÁ J., LUKEŠOVÁ A.: Morphological variability in selected heterocystous cyanobacterial strains as a response to varied temperature, light intensity and medium composition. *Folia Microbiol.* **53**, 333–342 (2008).