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Ahmed, M.; Stal, L.J.; Hasnain, S.

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REGULAR ARTICLE

Association of non-heterocystous cyanobacteria with crop plants

Mehboob Ahmed · Lucas J. Stal · Shahida Hasnain

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Abstract Cyanobacteria have the ability to form associations with organisms from all domains of life, notably with plants, which they provide with fixed nitrogen, among other substances. This study was aimed at developing artificial associations between non-heterocystous cyanobacteria and selected crop plants. We isolated several non-heterocystous cyanobacteria from various rice fields. The cultures were tested for their capacity to produce the plant hormone indole-3-acetic acid (IAA), and the possible role of IAA in the association of cyanobacteria with seedling roots was evaluated. Axenic cultures were co-inoculated with 10-day-old plant seedlings of *Triticum aestivum*, *Vigna radiata* and *Pisum sativum* and incubated for 1 week. Cyanobacterial association with the roots of these seedlings was quantified by measuring chlorophyll-*a*. Cyanobacterial association with the roots was observed by light microscopy as well as by confocal laser scanning microscopy (CLSM). Based on sequence analysis of the 16S rRNA gene, the isolates were identified as *Synechocystis* sp., *Chroococcidiopsis* sp., *Leptolyngbya* sp., and *Phormidium* sp. CLSM observations revealed the intimate association of cyanobacteria with the seedling roots as well as invasion of the roots and root cells. Strains producing IAA were more efficient in the colonization of the roots than those that lacked this ability. IAA-producing cyanobacteria possess a tryptophan-dependent pathway, and these cyanobacteria showed IAA synthesis activity in the presence of roots in media lacking tryptophan. Based on the results of this study, we conclude that non-heterocystous cyanobacteria also have the potential for use in agriculture to improve the growth and yield of crop plants that do not naturally form associations with cyanobacteria.

Keywords Non-heterocystous cyanobacteria · Artificial association · Indole 3-acetic acid · Confocal laser scanning microscopy

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M. Ahmed · S. Hasnain (✉)
Department of Microbiology and Molecular Genetics,
University of the Punjab,
Lahore 54590, Pakistan
e-mail: genetic@brain.net.pk

M. Ahmed · L. J. Stal
Department of Marine Microbiology,
Netherlands Institute of Ecology—KNAW,
PO Box 140, 4400 AC Yerseke, The Netherlands

Introduction

"No organism exists alone in nature and interactions between organisms are a rule rather than exception" (Rai et al. 2000). Cyanobacteria are a highly diverse, monophyletic group of *Bacteria* that may have existed as long as 3.5 billion years BP. Cyanobacteria are oxygenic phototrophic organisms that were responsible for the oxygenation of Earth's atmosphere 2.5 billion years ago, and which, through an endosymbiotic event,

gave rise to the chloroplasts in plants and algae. There is evidence that they entered into symbiotic relationships with some of the first land plants, such as liverworts and hornworts (Raven 2002).

Cyanobacteria occur in almost every environment on Earth and several species are known to form symbiotic associations with bacteria as well as with eukarya (Bergman et al. 2007). N_2-fixing cyanobacteria, particularly *Nostoc*, form symbiotic associations with a wide range of plants, such as angiosperms, as well as with algae, and provide fixed nitrogen to their host (Rai et al. 2000). Cyanobacteria may also occur as epiphytes (growing on the phyllo- or rhizosphere) on plants growing in aquatic and high-humidity environments (Freiberg 1999). However, there are no natural symbioses between cyanobacteria and crop plants (Rai and Bergman 2002). Many cyanobacteria are capable of N_2 fixation and, in the majority of cases, the function of the symbioses with algae and plants is to provide their host with fixed nitrogen. In symbioses with a chemotrophic host, symbiotic cyanobacteria may also provide the host with fixed carbon. The benefits for the cyanobacteria are much less obvious. The symbiont may obtain organic carbon or nutrients from the hosts, which may also protect them from predation or from environmental extremes, such as high light intensity or desiccation (Adams et al. 2006).

Cyanobacteria are also known for taking up and assimilating organic compounds, which may play an important role in secondary metabolism (Prasanna et al. 2004). Moreover, some cyanobacteria may produce plant growth-promoting substances (Sergeeva et al. 2002). There is currently great interest in developing new associations between cyanobacteria and crop plants (Nilsson et al. 2005; Rai and Bergman 2002). This might have important advantages for the establishment, growth and yield of crops and could minimize the need for fertilizers.

Hitherto the emphasis has been on the heterocystous Nostocaceae because the majority of the cyanobacteria that form natural symbiotic associations with plants belong to *Nostoc* (Gusev et al. 2002). Nilsson et al. (2002) tested numerous symbiotic cyanobacterial isolates of *Nostoc* for their efficiency of association with rice and found that, under laboratory conditions, some were successful. Svircev et al. (1997) also used *Nostoc* and *Anabaena* in order to develop new associations with various crops. Cyanobacteria are known to cohabitate with rice and proliferate as floating assemblages on the soil–water surface (Prasanna et al. 2009). Because most of this research has thus been done with rice, other economically important crops have been ignored.

In this study we investigated the potential of non-heterocystous cyanobacteria isolated from rice fields to form associations with three economically important crop plants other than rice. The cyanobacterial isolates were identified by their 16S rRNA gene sequences, growth on N_2, and production of the plant hormone indole-3-acetic acid (IAA).

Materials and methods

Sampling

Samples for enrichment and isolation of cyanobacteria were obtained from rice fields located on the campus of the University of the Punjab, New Campus, Lahore, Pakistan. Healthy rice plants were uprooted with attached rhizospheric soil. Excess soil was removed by gentle shaking. Roots with adhering rhizospheric soil were cut into pieces and immediately transferred to sterile Petri dishes. Water samples containing cyanobacterial growth were collected in sterile flasks. Samples were placed in an icebox and transported to the laboratory where they were stored at 4°C until use (within 1 week). Cyanobacteria were enriched in BG-11 medium (with or without 1.5 g l^{-1} $NaNO_3$; Rippka et al. 1979).

Enrichment and isolation of cyanobacteria

Isolation was started by suspending portions of the samples (root pieces and water samples) in BG11 medium. Subsequently, a series of ten-fold dilutions were made and spread on agarose-solidified BG-11 medium plates. Unicellular cyanobacteria were purified by repeated streaking on agarose medium (1% w/v) and by using the micropipette isolation technique, single colonies were transferred to fresh solid medium (Stal and Krumbein 1985). After viewing under the microscope, single colonies were transferred to liquid medium in flasks and grown with or without shaking. Filamentous cyanobacteria were isolated by cutting single trichomes from the solid medium using a sterile razor blade and transferring to a new plate. This process was repeated three to four times until axenic

filaments were obtained. The axenic condition of the cultures was tested by transferring pieces of agarose block with the trichomes to LB agarose medium plates. Finally, colonies were transferred to liquid medium using a loop or a fine tipped glass rod.

Co-cultivation of cyanobacteria and seedlings

Certified seeds of wheat (*Triticum aestivum* var Uqab-2000), pea (*Pisum sativum* var. Climax) and mung-bean (*Vigna radiata* var NM-92) were obtained from the Punjab seed cooperation, Lahore Pakistan. Seeds with no sign of physical or pest damage were selected as healthy seeds and their surface was sterilized by washing them for 5 min in 0.1% $HgCl_2$ followed by repeated washing with sterile distilled water. Sterile seeds were transferred to Petri dishes containing sterile Whatman filter paper No.1, and 5 ml sterile Milli-Q-Grade (MQ grade) water was added to each plate. Plates were kept in the dark to let the seeds germinate. After germination, the plates were incubated under fluorescent light (16:8 hour light: dark period) at a light intensity of 200 μE m^{-2} s^{-1}. Ten-day-old seedlings were suspended in 50 ml tubes with their roots immersed in 25 ml ten-fold diluted BG11 (N^+) or $BG11_0$ (N^-) medium. Eighteen-day-old cyanobacterial cultures were harvested by centrifugation at 10,000 *g* for 10 min and resuspended in 10 ml sterile MQ grade water. Cyanobacteria were added to the suspension of seedling roots to a final concentration of 2 μg chlorophyll-*a* (chl *a*) ml^{-1}. Incubation of the seedling roots and the cyanobacteria was carried out at 25°C under a 16 h:8 h light-dark cycle (200 μE m^{-2} s^{-1} light intensity). After 7 days of incubation, the seedlings were harvested and the roots were excised. Loosely attached cyanobacteria were removed by washing with sterile MQ grade water using a squeeze bottle, and the roots were subsequently used for assessing colonization by extraction of chl-*a*.

Chlorophyll-a determination and measurement of root dry weight

Cultures of cyanobacteria were harvested by centrifugation at 10,000 *g* for 10 min. The pellets were extracted by 80% methanol for 2 h in the dark at 4°C. The extract was centrifuged at 10,000 *g* for 10 min at 4°C, and the absorbance of the supernatant was measured spectrophotometrically at 665 nm against 80% methanol. Chl-*a* content (μg ml^{-1}) was determined from the optical density at 665 nm ($OD_{665\ nm}$), calculated as $OD_{665\ nm} \times 13.9$ (Tandeau de Marsac and Houmard 1988). Cyanobacteria associated with the roots were extracted with 80% methanol. The extracts were thoroughly vortexed. Roots from which the chl-*a* of associated cyanobacteria had been extracted, were subsequently dried for 72 h at 80°C and then weighed. The average of three independent experiments (triplicates) was taken (Nilsson et al. 2002).

Measurement of auxin production

The Salkowski colorimetric technique was used for the estimation of auxin in cultures of cyanobacteria containing plant roots (Glickmann and Dessaux 1995). Cultures were harvested by centrifugation at 5,000 *g* for 20 min at 4°C. Auxin concentration was also measured from medium containing only plant seedlings. Salkowski reagent was added to the supernatant in a ratio of 2:1 (v/v). Concentrations of auxin-like substances were estimated by absorbance at 530 nm after 30 min in the dark at room temperature against a control containing 1 ml culture medium and 2 ml Salkowski reagent.

Light microscopy

Roots containing cyanobacteria were observed using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). Micrographs were taken with an AxioVs40 and processed with V4.7.1.0 imaging software (Carl Zeiss Imaging Solution, Oberkochen, Germany).

Confocal laser scanning microscopy

Seedling roots were excised after 10 days of incubation with cyanobacterial isolates. To stain the root cells, excised roots (cut into pieces of 5 mm) were stained with fluorescent dye DTAF [5-(4, 6-dichlorotriazinyl) Aminofluores, Cat # D-16, Invitrogen, La Jolla, CA] by keeping samples in 1 mM DTAF solution overnight and by TRITC conjugated-*Cytisus scoparius* Lectin (CSA-TRITC), excised roots were stained with fluorescent dye by keeping samples in 1 mM CSA (Cat # R-3201-1, EY Laboratories, San Mateo, CA) solution in phosphate buffer, pH 8 for 20 min. Excess stain from the sample was removed

by washing with phosphate buffer twice, followed by carbonate buffer (pH 9) twice. Confocal laser scanning microscopy (CLSM) was performed using a TCS-NT microscope (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser. For simultaneous imaging of emission fluorescence from DTAF and auto fluorescence of cyanobacterial chl-*a* and phycobiliproteins, root sections were excited by beam of wavelength 488 nm. Emitted wavelengths from DTAF were collected using band-pass filter 530/30 and cyanobacterial natural fluorescence with a 590-nm-long pass filter. Images were obtained from the same field at different depths. To visualize the cyanobacterial extracellular colonization, the root surface was observed and a stack of images were generated until a depth of 5–10 μm, whereas for imaging the intracellular colonization the stack of images were obtained until a depth of 40–50 μm. The acquired images were analyzed with Leica TCS NT/SP SCANWARE (version 1.6.587) software. Overlaid images were generated by the outputs of two channels. Maximum projection algorithm, which determines the maximum of all intensity values in a stack of sections and displays them in one single image, was applied. Localization of cyanobacterial strains present intracellularly was analyzed by 3D rendering of the stack of images and their optical sectioning. All figures were produced and edited with Adobe Photoshop CS3 ver 10.0.1. The relative abundance of the cyanobacterial colonization (extracellular and intracellular) was analyzed by observing their instances on the root pieces of each plant type.

DNA extraction

A 15-day-old culture were used for DNA extraction. An aliquot of culture (10 ml) was centrifuged at 5,000 *g* for 15 min and the supernatant was discarded. With the help of a sterile loop, the pellet was transferred to a microcentrifuge tube provided with the UltraClean Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA). DNA extraction was performed following the instructions of the manufacturer, except that the initial vortexing step was increased to 15–30 min at maximum speed. DNA was eluted with 50 μl TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Amplification of the 16S rRNA gene

The 16S rRNA gene from the cyanobacterial isolates was amplified using forward primer (P16S 6 CYAN F) 5′ AGAGTTTGATCCTGGCTCAGG ′3 and reverse primer (p16S 1438cyanR) 5′ GGGCGGTGTGTACAAGG ′3. PCR reactions were performed in a 25 μl reaction volume. For each 25 μl PCR reaction, the mixture contained 0.1 μl (0.62 units) *Taq* polymerase (Qiagen, Venlo, The Netherlands), 1.0 μl of 5 mM dNTPs (Roche, Woerden, The Netherlands), 2.5 μl 10X PCR buffer (Qiagen), 0.5 μl (5 pmol) of the forward and reverse primers, 1 μl of template DNA (10–20 ng μl^{-1}) and 19.4 μl purified MQ-grade water (Millipore purification unit, 18 MΩ.cm; Millipore, Billerica, MA). The PCR program was run on a Gene Biometra T1 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) with the following settings: 2 min at 94°C, 35 cycles of 1 min at 94°C, 30 s at 55°C, 1.5 min at 72°C, and a final step of 10 min elongation at 72°C. PCR products were checked on a 1% agarose (Sigma Aldrich) gel. Amplicons were purified using the DNA clean and concentrator-5 kit (Zymo Research, Orange, CA) and eluted in 20 μl sterile MQgrade H_2O. DNA concentrations of purified PCR products were checked with the NanoDrop ND1000 spectrophotometer.

Cloning

Amplified PCR products were cloned into pCR 4-TOPO plasmids containing sites for universal primers T7 forward and T3 reverse using the TOPO™ TA cloning kit (Invitrogen, Breda, The Netherlands) according to the manufacture's instructions. Eight colonies containing the cyanobacterial 16S rRNA gene insert were picked for each PCR product and inoculated in microtiter plates (type 655180, Greiner Bio-one, Alphen a/d Rijn, The Netherlands) containing 50 μl sterile MQ-grade H_2O. The mixture was heated for 10 min at 95°C and subsequently used as template in a PCR reaction using the T7 and T3 primers provided with the TOPO cloning kit. The PCR program was a 15-min hot start at 94°C, 35 cycles of 1 min at 94°C, 30 s at 55°C, 1.5 min at 72°C and a final step of 7 min elongation at 72°C. The PCR products were checked on a 1% agarose gel.

Purification

PCR products were purified using the DNA clean and concentrator-5 kit (Zymo Research) and eluted in 20 µl sterile MQ grade H_2O. DNA concentrations of purified PCR products were checked with the Nano-Drop ND1000 spectrophotometer.

Sequencing

Four different sequencing reactions were performed for each strain, i.e., one set of forward and reverse primers as mentioned above and one set of internal primers (forward primer (CyaP2) 5′ CCGGAA-TIATTGGGCGTAA ′3 and reverse primer (16Sm1052Cyap5) 5′ CTCGTTGCGGGACT-TAACCC ′3). The PCR program was run on a Gene Biometra T1 DNA Thermal Cycler (Perkin-Elmer) with the following settings: 15 min hot start at 94°C, 25 cycles of 30 s at 96°C, 15 s at 50°C, 2 min at 60°C

The sequencing reactions were performed by using the Big Dye Terminator v1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) following the instructions of the manufacturer. Sequence products were analyzed with a 3130 Genetic Analyzer (Applied Biosystems). For each strain, the forward, reverse and internal sequences were edited, aligned and assembled using CLC DNA Workbench Software. The consensus sequences were checked against GenBank using BlastN (Altschul et al. 1997; McGinnis and Madden 2004).

Statistical analysis

Data were analyzed statistically using SPSS personal computer statistical package (version 16, SPSS, Chicago, IL). Analysis of variance (ANOVA) was performed and then means were separated using Duncan's multiple range test (P=0.05).

Results

Isolation and purification of cyanobacteria

Cyanobacteria were isolated from different locations of the agricultural areas of Quaid-e-Azam campus, University of the Punjab, Lahore, Pakistan. This area is continuously cultivated with wheat and rice crops. Strains were isolated from different field sites, i.e., from plant rhizosphere and free-living forms present in water, and for compared for their colonization ability. The selected isolated strains showed differences in their characteristics as summarized in Table 1. The characterization and assignment of the isolated cyanobacteria was done according to *Bergey's Manual of Systematic Bacteriology* (Boone et al. 2001), which basically follows the classification of Rippka et al. (1979). Three isolates are unicellular; MMG-5 and MMG-6 (*Chroococcidiopsis* sp.) (Subsection II) and MMG-8 (*Synechocystis* sp.) (Subsection I), while strains MMG-1 (*Leptolyngbya* sp.) and MMG-4 (*Phormidium* sp.) are filamentous strains belonging to subsection III. We did not isolate heterocystous cyanobacteria but nevertheless three strains grew in media without a source of combined nitrogen (nitrate) and exhibited nitrogenase activity when measured by ARA (*Leptolyngbya* sp. MMG-1 and the unicellular *Chroococcidiopsis* sp. MMG-5 and MMG-6) (Table 1).

Genetic identification

Sequences of the 16S rRNA gene were compared with the NCBI sequence database (GenBank) through BLAST (www.ncbi.nlm.nih.gov/BLAST). On the basis of sequence identity, strain MMG-1 was most closely related to *Leptolyngbya* sp. CENA103 with 97% similarity. Strain MMG-4 was closest to *Phormidium* cf *terebriformis* KR2003/25 with 99% similarity, while strains MMG-5 and MMG-6 were 99% and 97% similar, respectively, with *Chroococcidiopsis* sp. PCC 7431, and strain MMG-8 was 99% similar with *Synechocystis* sp. PCC 6803. The 16S rRNA gene sequences were submitted to NCBI GenBank under accession numbers FJ839352, FJ839355, FJ839356, FJ839357 and FJ839359 for strains MMG-1, MMG4, MMG-5, MMG-6 and MMG-8, respectively.

Colonization of plant roots

Three crop plants, wheat, mungbean and pea, were used. All five cyanobacterial strains investigated in this experiment were able to colonize seedling roots but to different extents. The colonization of plant roots by cyanobacteria is shown in Table 2. Colonization was defined as cyanobacterial biomass (Chl-*a*)

Table 1 Some morphological and biochemical characteristics of the isolated strains. *R* Round, *BG* blue-green, *DG* dark green, – absent, + present

Strain	Characteristics											
	Shape	Cell width (μm)	Hormogonia	Cell color	Sheath	Gas vesicle	Baeocyte	Heterocyst	Nitrogenase activity (μmol C_2H_4/g Chl-a)[a]	Motility[b]	Auxin production	Subsection
MMG-1	Filamentous	1–2	+	BG	+	+	–	–	0.645	–	+	III[c]
MMG-4	Filamentous	6–7	+	DG	+	+	–	–	–	+	–	III[c]
MMG-5	Unicellular, aggregation	3–4	–	DG	+	–	+	–	0.95	–	+	II[c]
MMG-6	Unicellular, aggregation	3–4	–	DG	+	–	+	–	0.763	–	–	II[c]
MMG-8	Unicellular	3	–	BG	+	–	–	–	–	+	+	I[c]

[a] Measured by ARA (Hardy et al. 1968) after 24 h of incubation

[b] Motility here refers to cells or filaments, not to hormogonia, which are always motile

[c] Following *Bergey's Manual of Systematic Bacteriology* (Boone et al. 2001)

on the roots after a certain time (1 week) of incubation that resisted washing off with distilled water from a squeeze bottle. The level of colonization was quantified by measuring the chl-*a* content tightly bound to the roots after a week of incubation (Nilsson et al. 2002).

In the presence of nitrate-supplemented medium, both filamentous strains showed significantly higher association with wheat as compared to the other two plants. Maximum association (978 μg chl-*a* g^{-1} root dry wt) was observed in the case of *Leptolyngbya* sp. MMG-1 with wheat roots, whereas with mungbean and pea the chl-*a* value was significantly lower (40%). Similarly, *Phormidium* sp. MMG-4 associated better with wheat than with other plants. The unicellular strains MMG-5 and MMG-6 associated less with wheat and showed better association with pea (50% higher than with wheat) and mungbean (40% higher than with wheat). The unicellular strain *Synechocystis* sp. MMG-8 showed low associations (average 80 μg chl-*a* g^{-1} root dry wt) with any of the plants. In nitrate-deficient media, non-fixers, i.e., *Phormidium* sp. MMG-4 and *Synechocystis* sp. MMG-8, could not grow, so were unsuccessful. Maximum chl-*a* (815 μg chl-a g^{-1} root dry wt) was observed in the case of *Chroococcidiopsis* sp. MMG-5 with pea roots. *Chroococcidiopsis* spp. MMG-5 and MMG-6 showed better affinity with pea and mungbean (average 20% and 50% higher, respectively) in nitrate-deficient medium as compared to nitrate supplemented medium. *Leptolyngbya* sp. MMG-1 showed similar results with pea and mungbean but in the case of wheat there was significantly lower (21%) chl-*a* content (Table 2).

Light microscopy

Chroococcidiopsis sp. MMG-5 and MMG-6 colonized root surfaces of all three plants. In the case of strain MMG-5, the association was much tighter, i.e., forming a mat-like growth (Fig. 1a) especially with leguminous plants (mungbean and pea). These cyanobacteria also invade the epidermal cells and colonize the root cells. In contrast, strain MMG-6 forms a loose association with the root surface and sticks mostly to root hairs (Fig. 1b), *Synechocystis* sp. MMG-8 preferred growth in the free-living state rather than growth in associations with roots, although this organism still inhabited wheat roots and in some

Table 2 Cyanobacterial colonization of plant roots, measured as chlorophyll-*a* (chl-*a*) in root dry weight (μg chl-a g^{-1} root dry wt) Mean of three independent experiments ± standard error. Different letters indicate significant differences between columns using Duncan's multiple range test (P=0.05). These values represents the maximum extractable chl-*a* content that includes both extracellular and intracellular colonization

	Mung bean		Pea		Wheat	
	N+	N–	N+	N–	N+	N–
MMG-1	556±2.33 d	580±2.65 b	543±2.20 d	550±3.68 b	978±5.54 e	768±3.65 d
MMG-4	347±1.54 b	1.5±0.11 a	310±0.87 b	3±0.23 a	612±3.12 d	4±0.16 a
MMG-5	614±3.32 e	815±4.77 c	701±2.76 e	783±5.45 c	432±4.32 c	440±3.31 c
MMG-6	457±3.10 c	777±4.96 c	498±2.20 c	750±3.44 c	333±2.42 b	374±2.22 b
MMG-8	69±0.56 a	2±0.08 a	77±1.20 a	0±0.00 a	87±1.38 a	3±0.12 a

cases was found even inside the epidermis cells. *Leptolyngbya* sp. MMG-1 grew freely and covered the roots, preferring wheat roots and particularly the apical parts, while *Phormidium* sp. MMG-4 also surrounded roots but did not cover the apical tips (Fig. 1c,d).

Confocal laser scanning microscopy

CLSM revealed that *Phormidium* sp. MMG-4 grew only on the root surface, covering and spreading in the spaces between the epidermal cells (Fig. 2a). This cyanobacterium attached to the roots of all three

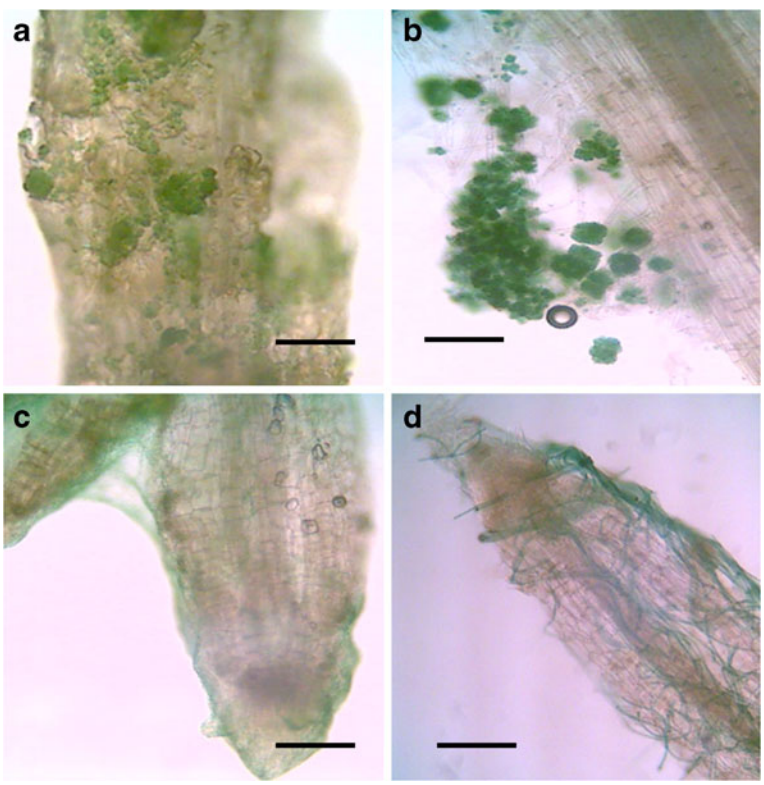

Fig. 1 a–d Light microscopy of root sample colonized by cyanobacterial isolates. **a** *Chroococcidiopsis* sp. MMG-5 colonizing mungbean roots. **b** *Chroococcidiopsis* sp. MMG-6 growing in mungbean pea root hairs. **c** *Leptolyngbya* sp. MMG-1 growing and spreading on wheat roots. **d** *Phormidium* sp. MMG-4 spreading on wheat root. *Bars* **a–c** 20 μm; **d** 50 μm

plants but showed a somewhat higher affinity for wheat (Table 3). *Leptolyngbya* sp. MMG-1 not only grew on the root surface but also invaded the cells. This cyanobacterium showed also more interaction with wheat than with the other plants (Table 3). Filaments entering one cell started colonizing the same cell and also spreading to adjacent cells (Fig. 2c). *Chroococcidiopsis* sp. MMG-5 colonized the root surface and ridges in cells and also invaded the epidermis and below (Fig. 2b). *Chroococcidiopsis* sp. MMG-6 remained outside the root surface. Both of these unicellular strains preferred mungbean and pea and were less attracted to wheat (Table 3). Strain MMG-8 colonized the root surface, and in the case of mungbean it also infected the epidermal cells and grew inside the root cells (Fig. 2d).

IAA secretion

Auxin was measured in culture media after incubation with plant roots. Only three strains, *Leptolyngbya* sp. MMG-1, *Chroococcidiopsis* sp. MMG-5 and *Synechocystis* sp. MMG-8, showed the ability to synthesise auxin. A significantly higher amount of auxin (average 25 $\mu g ml^{-1}$) was detected with *Chroococcidiopsis* sp. MMG-5 with all three plants and in both nitrate-supplemented and nitrate-free medium (Fig. 3). *Leptolyngbya* sp. MMG-1 produced more auxin in nitrate-deficient medium than in nitrate-supplemented medium. Incubation with mungbean roots resulted in significantly higher amounts of auxin production (Fig. 3). *Synechocystis* sp. MMG-8 did not grow in nitrate-deficient medium, and auxin production was higher with mungbean roots compared to wheat and pea (Fig. 3). No auxin was detected in media containing seedlings without cyanobacteria.

Discussion

Cyanobacteria can occupy almost any environment on Earth, which includes not only illuminated places but also many dark places such as rock crevices and in the soil (Furey 2003). Their presence in soils is of great importance for the functioning of many processes. Cyanobacteria present in the rhizosphere are hugely

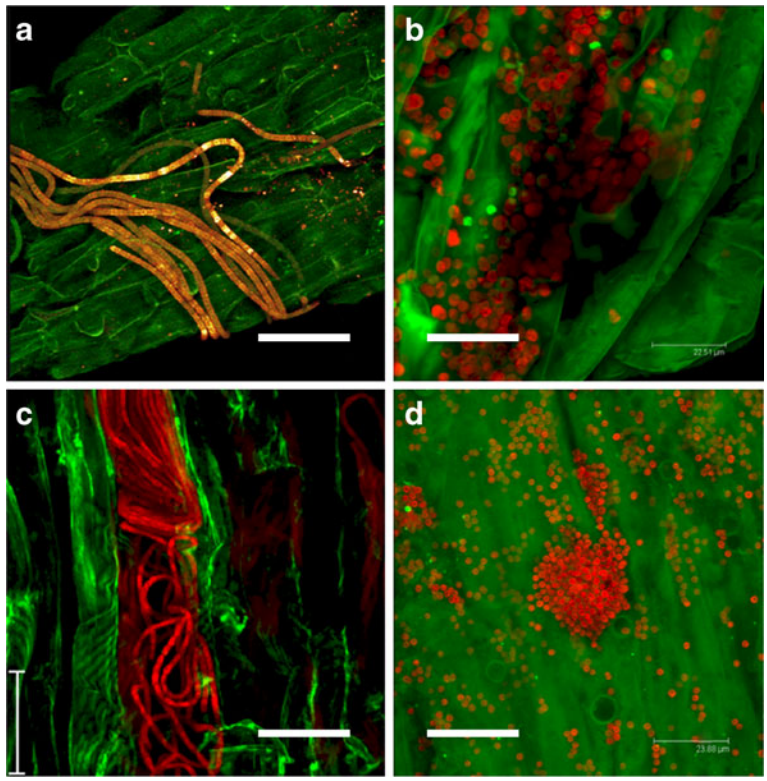

Fig. 2 **a**–**d** Confocal laser scanning microscopy (CSLM) of root sample colonized by cyanobacterial isolates. **a** *Phormidium* sp. MMG-4 spreading on pea root. **b** *Chroococcidiopsis* sp. MMG-5 invading inside mungbean root cells. **c** *Leptolyngbya* sp. MMG-1 growing and spreading in wheat root epidermal cells. **d** *Synechocystis* sp. MMG-8 colonizing wheat root surface and inside cells. *Bars* **a** 20 μm, **b** 23 μm, **c** 50 μm, **d** 30 μm

Table 3 Comparison of exogenous and endogenous colonization by cyanobacterial isolates in three plants based on observation under confocal laser scanning microscopy (CLSM)

Strain	Nitrate	Mung		Pea		Wheat	
		Exogenous	Endogenous	Exogenous	Endogenous	Exogenous	Endogenous
MMG-1	N+	++[a]	+	+	+	++	+++
	N–	+	+	+	+	+	+
MMG-4	N+	+	–	+	–	++	–
	N–	–	–	–	–	–	–
MMG-5	N+	++	+	+	++	+	–
	N–	+++	+	++	+	+	+
MMG-6	N+	++	–	++	–	+	–
	N–	+++	–	++	–	+	–
MMG-8	N+	+	+	+	–	+	–
	N–	W+	–	–	–	W+	–

[a] +++ Highest, ++ higher, + low, W+ weak, – no association

important but largely ignored (Prasanna et al. 2009). This group of organisms is particularly beneficial for the plants associated with them. Remarkably, hitherto no natural symbiotic associations between cyanobacteria and crop plants have been reported (Rai and Bergman 2002). This study aimed to investigate the potential of cyanobacteria to form associations with different crop plants. In order to achieve this goal we isolated cyanobacteria from agricultural fields (i.e., rice paddies) under different conditions. Cyanobacteria are common inhabitants of agricultural areas, particularly rice fields. In most cases, soil cyanobacteria exhibit great biodiversity. Filamentous forms always dominate the biomass of cyanobacterial population present in soil and rice fields (Whitton 2000).

Two approaches are generally used to study cyanobacterial association with roots. Chlorophyll-*a* is measured to give a quantitative estimation, which only gives the extent of association irrespective of the location and type of attachment, while for qualitative examination or type of association i.e. endogenous or exogenous, imaging techniques such as light microscopy or scanning electron microscopy are used. During the incubation of the tested crop plants with the cyanobacterial isolates, three categories of interaction could be distinguished by microscopy. Firstly, colonization with tight association followed by the penetration of the cyanobacteria into the root epidermal cells (*Leptolyngbya* sp. MMG-1 and *Chroococcidiopsis* sp. MMG-5 with all three plants). Secondly, a tight association without intracellular penetration (all cyanobacteria with mung bean). Thirdly, simple colonization with only loose attachment to the root surface and root hairs [*Chroococcidiopsis* sp. MMG-4 with all tested plants (Fig. 1b) and Synechocystis sp. MMG-8 on wheat roots].

Leptolyngbya sp. MMG-1 grew without combined nitrogen and exuded auxin when incubated with roots. This cyanobacterium expressed a type 1 association both in nitrate-supplemented and nitrate-deficient media. The maximum association of strain MMG-1 was with wheat, irrespective of whether it was grown with or without nitrate. With the other two test plants, i.e., mung and pea, the extent of association remained the same and there was no significant difference

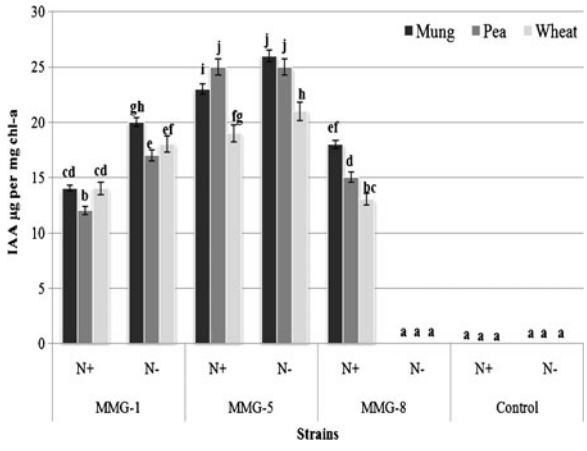

Fig. 3 Auxin production by isolates after coinoculation with seedlings. Values are mean of three independent measurements with *standard error bars*. Letters on each bar followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test

between chl-*a* values. *Phormidium* sp. MMG-4 also preferred wheat. This organism was unable to grow in nitrate-deficient media. The association of strain MMG-4 with the roots was of type 2 since the cyanobacterial trichomes spread only on the root surface (Fig. 2a). Associations between filamentous cyanobacteria and different crop plants, such as with rice (Nilsson et al. 2002), wheat (Gantar 2000), corn, sugarbeet and bean (Svircev et al. 1997), have been reported previously. By using scanning electron microscopy (SEM), Gantar et al. (1991) reported intercellular and intracellular associations of *Nostoc* sp. with wheat roots, whereas Nilsson et al. (2002) demonstrated the intercellular presence of *Nostoc* sp. in rice root epidermis. In this study, we applied CLSM to investigate the intracellular presence of *Leptolyngbya* sp. MMG-1 in wheat epidermal cells (Fig. 2b). This strain fully occupied the apparently empty plant cells and afterwards started spreading to adjoining cells (Fig. 2c).

The unicellular strain *Chroococcidiopsis* sp. MMG-5 forms a type 1 association with the roots of all three plants used, whereas *Chroococcidiopsis* strain MMG-6, isolated from free growth in field water, showed only exogenous colonization (type 3 association). According to measurements of chl-*a* value from roots, both MMG-5 and MMG-6 grew in nitrate-depleted media and associated equally well with all three plants. Strain MMG-5 also produced auxin in the culture medium, which also indicates its association as being more endogenous with roots through induction of paranodules (Nilsson et al. 2002). Mung bean, being a leguminous plant, is highly attractive to rhizospheric microbes, abundantly secreting components such as polycyclic aromatic compounds known as flavonoids and betaines. These compounds acts as strong attractants for microbes and stimulate their major plant growth-promoting activities such as associative nitrogen fixation, IAA synthesis, EPS formation and their colonization ability (Mathesius 2008).

In nitrate-supplemented medium, *Synechocystis* sp. MMG-8 did not associate with roots except to form loose type-3 associations with mungbean and in a very few cases it was even present intracellularly. Unicellular cyanobacteria occur in natural symbioses with some diatoms and lichens (Rai et al. 2000; Schenk et al. 1992). *Synechococcus* sp. PCC 6301 has been located on the surface as well as in the interior of plant cell aggregates in suspension culture (Gusev et al. 2002). Usually, cyanobacteria in natural symbioses contain specific sugars, pili, and, in some cases, lectins, which are involved in the recognition of, and adhesion to, a compatible host (Rai et al. 2000). However, the exact mechanism by which these cyanobacteria are able to enter plant cells is still unknown (Adams et al. 2006; Gusev et al. 2002).

Five distinct phases were clearly seen in the association between filamentous cyanobacteria strain *Leptolyngbya* sp. MMG-1 and plant roots (Fig. 4a). In the first step, filaments enter into root cells. The exact mechanism of cyanobacterial penetration into root cells is still not known (Adams et al. 2006), but it is generally believed to be facilitated by hydrolytic enzymes of bacterial origin (Gantar et al. 1991). In the second step, after entering the plant cell, cyanobacterial filaments start spreading to surrounding cells, and in the third step they grow and multiply within the plant cell. In the fourth step, it seems that cyanobacteria rupture the plant cell wall by exerting mechanical pressure, and thus enter the neighbouring cells (Fig. 4b). In the fifth and last step, cyanobacterial filaments completely occupied the plant cell (Fig. 4c).

Cyanobacteria associated with plant cells are in direct contact with each other and both organisms synthesize a variety of biologically active substances that initiate and maintain the association (Bais et al. 2006). There are many reports of auxin production by cyanobacteria, mainly referring to indole-3-acetic acid (IAA), which is generally synthesized through a tryptophan-dependent pathway (Sergeeva et al. 2002; Tarakhovskaya et al. 2007). Auxin-like substances were determined in the culture media by Salkowski reagent. This reagent is considered as a simple and fast method of detecting indolic compounds such as IAA (Glickmann and Dessaux 1995). Although a less sensitive method of IAA determination, and although values are always higher than measurement by GC-MS, there is nevertheless a strong correlation between measurements using these two methods (Ali et al. 2009).

Among the cyanobacterial isolates used in this study, *Leptolyngbya* sp. MMG-1, *Chroococcidiopsis* sp. MMG-5 and *Synechocystis* sp. MMG-8 produce IAA-like substances in medium supplemented with L-tryptophan. However, during incubation with plant roots, auxin was also detected in the culture superna-

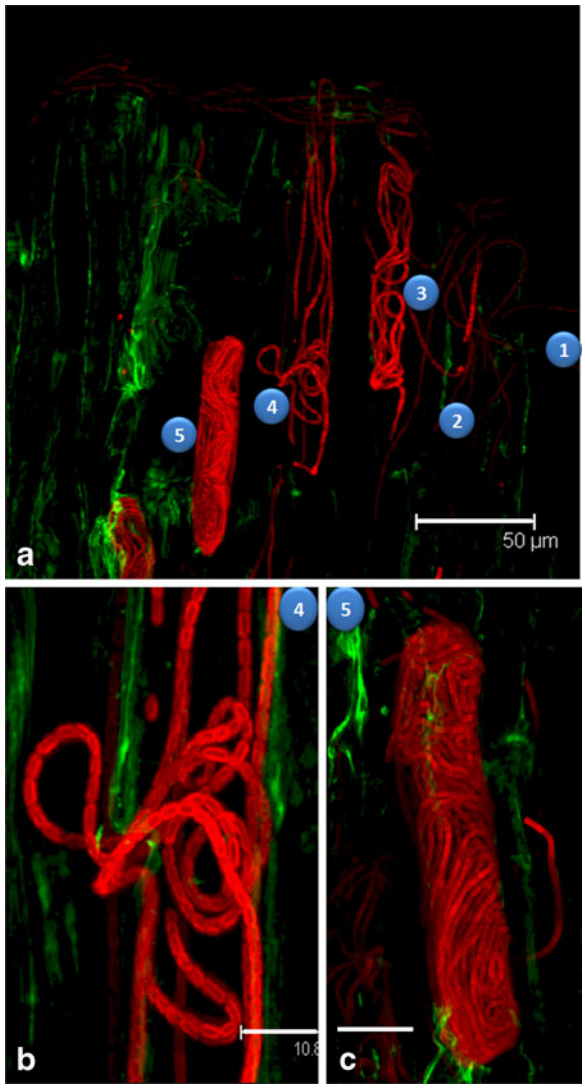

Fig. 4 **a**–**c** CLSM of root sample colonized by *Leptolyngbya* sp. MMG-1. **a** Different phase of association. **b**, **c** Enlarged phase 4. *Bars* **a** 50 µm, **b** 10 µm, **c** 20 µm

tant in the absence of added tryptophan. Root exudates are complex mixtures of various organic substances, including sugars, aminoacids, vitamins and other metabolites, which play a vital role in signalling with the rhizospheric community (Bais et al. 2006). However, the amount of auxin in root exudates is negligible, making it too hard to determine (Kamilova et al. 2006). Hence, it remains possible that the amino acid tryptophan was present in the root exudates, although other factors may also have played a role in stimulating auxin production in these cyanobacteria. Auxin detected in culture medium with mungbean was significantly higher than with the other plants with all three auxin-producing cyanobacteria. *Leptolyngbya* sp. MMG-1 and *Chroococcidiopsis* sp. MMG-5 both grew in nitrogen-depleted medium, and both strains produced more auxin in nitrogen-depleted medium than in nitrogen-sufficient medium. The third auxin producing strain, *Synechocystis* sp. MMG-8 grew only in nitrogen-supplemented medium. No auxin was detected in control conditions in media containing seedlings without cyanobacterial inoculation. Auxin is considered as a signalling substance that facilitates communication between associated partners. Auxin is known to not only significantly affect rooting in terms of root number and density but also to regulate cyanobacterial growth (Adams et al. 2006; Ahmad and Winter 1968). The synthetic auxin 2,4-D is reported to have a positive impact on cyanobacterial colonization of wheat roots through the formation of paranodules (Gantar and Elhai 1999). This may be a reason why the auxin-producing strain *Chroococcidiopsis* sp. MMG-5 showed better association with the tested plants compared to the non-auxin-producing strain *Chroococcidiopsis* sp. MMG-6 in nitrate-supplemented media. The other factor may be the origin of both strains, MMG-5 was isolated from the rhizosphere while MMG-6 originated from the water inundating the fields. But previous reports suggested that free-living strains also showed a higher level of root colonization (Nilsson et al. 2002).

In the majority of filamentous cyanobacteria, specialized cells—the heterocysts—are present, which provide a suitable environment for nitrogenase activity by withdrawing photosystem II and developing a thick glycolipid cell wall as a gas diffusion barrier to limit the influx of O_2 (Walsby 2007). By contrast, non-heterocystous cyanobacteria develop intercellular localization of nitrogenase enzyme and/or nocturnal nitrogenase activity to protect nitrogenase from the photosynthetic O_2 (Ohki 2008; Compaoré and Stal 2009). As plant roots are non-photosynthetic and consume any oxygen present in their vicinity by respiration, they thus create a micro-oxic environment in their surroundings. By forming associations with plant roots, non-heterocystous diazotrophic cyanobacteria utilize these conditions for their nitrogenase activity (van Heerden et al. 2008). Furthermore, plant roots secrete chemical signals such as flavonoids, that facilitate the nitrogenase activity (Theunis et al. 2004;

Mathesius 2008). This is indeed one possible reason why cyanobacterial isolates invade root tissues and pack themselves into the cells. A significant increase in cyanobacterial associative nitrogenase activity in the presence of plant roots demonstrates the functional significance of such close associations (Nilsson et al. 2002; Svircev et al. 1997). Formation of such sustainable symbiotic associations is the ultimate goal of cyanobacterial colonization with plant roots.

Conclusion

Rhizospheric and soil non-heterocystous cyanobacteria are a much ignored group of microbes that have the potential to form associations with the roots of crop plants, although this has hitherto not been reported from the natural environment. These cyanobacteria colonize the rhizoplane but also infect epidermal cells and invade and multiply intracellularly. Possible benefits for cyanobacteria include a favorable environment for nitrogen fixation. It appeared that non-symbiotic non-heterocystous cyanobacteria can form associations with different crop plants but the extent and nature of the relationships depends on both the strain and the plant species. There is a need to test more cyanobacterial strains for artificial symbiosis. Such cyanobacteria may be applied in the field as soil inoculants in combination with fertilizers.

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