Analysing the colonisation of inoculated cyanobacteria in wheat plants using biochemical and molecular tools

Santosh Babu · Radha Prasanna · Ngangom Bidyarani · Rajendra Singh

Abstract The modulation in physiological and metabolic attributes associated with colonisation by cyanobacteria in wheat has been little explored. In the present investigation, the performance of six selected cyanobacterial strains was evaluated with wheat (variety HD2687). The fresh weight of plants, measured after 2 weeks, exhibited a 30–60 % increase, while 14–40 % increase in plant dry weight was also recorded, as compared to uninoculated control. The nitrogen-fixing potential (expressed as acetylene-reducing activity or ARA) was 20-fold higher in the treatment involving inoculation of *Anabaena laxa* RPAN8 as compared to that in the uninoculated control. The inoculation of *Calothrix* sp. RPC1 brought about a more than 90 % increase in endoglucanase activity and root chlorophyll. Comparison of DNA fingerprints (highly iterated palindrome (HIP)-TG profiles) of wheat roots with those of corresponding pure cultures revealed a high degree of similarity, confirming the colonisation. Significant correlation of plant parameters with nitrogen-fixing potential and growth attributes and fingerprints of cyanobacteria from roots further illustrated the novelty of our results. This represents a first report on understanding hydrolytic enzyme-mediated colonisation of cyanobacteria on roots of wheat plants using plant growth parameters and DNA fingerprints. Such synergistic combinations of cyanobacterium and wheat can lead to savings of nitrogen and increased yields, besides being a prelude to generating nitrogen-independent wheat plants.

Keywords Chlorophyll · Cell wall hydrolysis · Cyanobacteria · Enzyme activity · Nitrogen · Wheat

Introduction

Wheat (*Triticuma estivum* L.) contributes about 33.5 % to the total world food grain production and represents one of the major sources of calories and protein in the human diet. India ranks second in the world in terms of acreage (12.5 %) and production (12.05 %) in intensive cropping systems (www.agricoop.nic.in). Crop productivity has been traditionally enhanced using chemical fertilisers in modern agriculture, but their low efficiency mainly due to losses through denitrification, volatilisation, leaching or immobilisation in soil has necessitated the need for identifying viable alternatives for use as nutrient supplements.

It is generally understood that plant growth promotion is dependent upon nutrient acquisition or stimulation, mainly mediated by associative microorganisms (Dobbelaere et al. 2003). Plant-growth-promoting rhizobacteria (PGPR) represent mainly soil bacteria growing in association with plants, which stimulate growth by increased mobilisation, uptake and enrichment of micronutrients and macronutrients in the plant. As a result, they can indirectly lead to bio-control of pathogenic flora/fauna or other competing native organisms for space or nutrients (Lucas García et al. 2004; Çakmakçi et al. 2006). Nitrogen is unique among the essential mineral elements in the plants, present in both anionic and cationic (NO_3 and NH_4) forms, and supplying the plant with a mixture of NO_3 and NH_4 often results in better vegetative growth and enhanced nutrient accumulation than either form separately (Hageman 1984).

S. Babu · R. Prasanna (✉) · N. Bidyarani
Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi 110012, India
e-mail: radhapr@gmail.com

R. Singh
National Phytotron Facility, Indian Agricultural Research Institute (IARI), New Delhi 110012, India

Enhancement of rice seed germination, root and shoot growth and the fertilising action of N_2 fixed from the atmosphere by free-living cyanobacteria has been illustrated in several studies. This generally leads to the release of synthesized nitrogenous compounds either by decomposition of the cells or excretion (Venkataraman and Neelakantan 1967; Nayak et al. 2004; Prasanna et al. 2008). In symbiotic associations, the nitrogen fixed by the cyanobiont is made available to the system, which in turn is supplied by the host with a niche for its growth and survival (Rai 1990). Due to the difficulty in maintaining specific N ratios in soil and its buffering capacity, much of this evidence has been obtained by growing plants hydroponically. Such studies indicate that vegetative growth of wheat is enhanced when both NO_3 and NH_4 are present (Weissman 1951) leading to higher total protein content and protein concentration in leaves of young wheat plants grown in solution culture containing NH_4 and NO_3 than on either form alone. Similarly, an enhancement (74 %) was observed in vegetative dry weight of 16-day-old wheat plants when NH_4 was supplied at the rate of 17 % of total N, as compared to plant grown with only NO_3 (Cox and Reisenauer 1973).

Inoculation with cyanobacteria can lead to yield improvement of 5–25 % not only in cereals such as rice and wheat but also in solanaceous fruits including tomato and in legumes and cotton (Prasanna et al. 2008, 2009, 2013a; Karthikeyan et al. 2009; Nain et al. 2010). Cyanobacteria represent promising organic inputs in rice-wheat cropping system, as they help in N and C accretion and improve crop productivity and soil health; the colonisation and establishment of inoculated cyanobacteria have been demonstrated using DNA-based markers (Prasanna et al. 2012). Earlier studies have shown that sugars and amino acids may serve as signalling molecules in the development of functional associations between relevant cyanobacteria and wheat seedlings (Jaiswal et al. 2008; Karthikeyan et al. 2009). Kovtunovych et al. (1999) found a high degree of correlation between the activity of cell wall depolymerising enzyme pectate lyase and internal colonisation of wheat roots by *Klebsiella* spp.; however, the role of endoglucanases, especially in cyanobacteria, is less investigated. Such studies can be particularly useful for developing nitrogen-fixing cyanobacteria associated wheat plants, as current research is being directed for genetic engineering of *nif* genes in cereals.

It is very well understood that considerable expertise is required to identify cyanobacteria, and studies on *Anabaena* species have revealed information that both morphological and developmental characters can vary with the growth conditions, particularly the shape and size of the cell/thalli, which are the basis for the classification and taxonomy of *Anabaena* (Hiroki et al. 1998). However, the use of DNA-based attributes is getting significant popularity in the identification of cyanobacteria at both generic and species levels. Filamentous

heterocystous cyanobacteria, including *Anabaena*, are known to have several distinct families of highly conserved repetitive sequences, such as short tandemly repeated repetitive (STRR) sequences and long tandemly repeated repetitive (LTRR) sequences, which have been used in combination with restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) typing (Rasmussen and Svenning 1998; Nayak et al. 2009) to distinguish the isolates at both inter- and intra-generic levels. Another set of interspersed repeated sequences, namely highly iterated palindrome (HIP) sequences, have proved valuable in the identification of differentiation of strains (Gupta et al. 2012). Such repeat/palindrome sequences, as a result of their conserved status, represent methodologically important tools for DNA fingerprinting as well as for diversity studies among related cyanobacteria.

In order to understand the chemical and biological nature of interactions of cyanobacterial strains with wheat seedlings, a hydroponics experiment was taken up at the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The strains used in the experiment had been earlier characterised for their nitrogen-fixing and PGP abilities (Nain et al. 2010; Prasanna et al. 2008, 2013b), and in the present study, efforts were undertaken to analyse the plant-cyanobacterium associations using biochemical/physiological attributes and employing molecular tools to illustrate colonisation of inoculated organisms in root tissues based on HIP-TG profiles and 16S ribosomal DNA (rDNA) sequences.

Materials and methods

Cultivation and maintenance of selected cyanobacterial strains

Cyanobacterial cultures used in study—*Anabaena torulosa* BF1, *Anabaena laxa* C11, *Anabaena azollae* C16, *Anabaena oscillarioides* C20, *Calothrix* sp. RPC1 and *A. laxa* RPAN8—were maintained by using standard protocols and cultivated in Haffkine flasks, in nitrogen-free BG11 medium (Stanier et al. 1971) at 27 ± 1 °C and light/dark cycles (L/D) of 16:8 and white light (50–55 μmol photons m^{-2} s^{-1}) as stationary cultures.

Experimental setup

An experiment with wheat and selected cyanobacterial strains was undertaken under controlled conditions, including five replicates of each treatment in the National Phytotron Facility (NPF), IARI, New Delhi. The experiment was carried out in 4-in pots containing 300 mL of autoclaved nitrogen-free BG11 liquid medium. Seeds (*T. aestivum;* variety HD2687) were sterilised with 0.1 % mercuric chloride for 1 min followed by washing with sterile water. Seeds were imbibed overnight in sterile water in dark at room temperature and kept for

germination for 24 h. A set of pots containing 300 mL of autoclaved chemically defined BG11 liquid medium was used as control. A thermocol plate with four 1.5-mL Eppendorf tubes containing overnight 24-h soaked seeds was adjusted on each pot individually. The base of each tube was cut to permit the emergence and growth of seedlings in pots. According to designed treatments for this study, the selected strains were inoculated with 1 mL of packed cell volume of each strains individually (approximately 10 μg chlorophyll mL^{-1} media) and grown for 14 days at 22±1 °C (day) and 18±1 °C (night), light intensities of 570 μmol photons m^{-2} s^{-1} for 3 h during peak day hours and 320 μmol photons m^{-2} s^{-1} in the beginning and end of the day for 1 h, with prominent red and far-red spectrum ratio 5:2, 0.6 % m s^{-1} air flow (bottom to top) and humidities of 45±1 % (day) and 55±1 % (night).

The seedlings were transferred to the pots, and after 15 days, the plant samples from each treatment were harvested for the analyses.

Plant-growth-promoting attributes

Fresh and dry weights of plants were measured as growth indices. Chlorophyll content of plants (shoot and root) and BG11 medium inoculated with selected cyanobacterial strains were estimated following standard methods (Mackinney 1941; Daizy and Kohli 1991) using 95 % methanol. Nitrogenase activity of plants was measured by acetylene reduction activity (ARA) by using Bruker 450-gas chromatograph (GC) with FID detector. For the analysis of nitrogenase activity, plants were placed in glass test tubes (55 mL) and sealed with airtight rubber stoppers followed by replacement of 10 % air space (v/v) with acetylene. The tubes were incubated for 24 h. A set of tubes without acetylene gas injection served as control. All experiments were carried out in triplicates. After incubation, 0.1 mL of gaseous sample was taken tubes and injected into GC for analysis. The ethylene produced by the reduction of acetylene was estimated and expressed as nanomoles of ethylene produced per plant per hour.

Activity of hydrolytic and defence enzymes in seedlings

Root tissues were cleaned by running tap water and homogenised with pre-chilled 50 mM (5 mL) Tris-HCl buffer in a mortar pestle. The homogenous extracts were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was collected and stored at −20 °C. Polyphenol oxidase (PPO) and peroxidase (PO) activities were analysed using catechol and 1 % guaiacol (molar extinction coefficient 26.6 mM cm^{-1}) as substrate by minor modifications (Prasanna et al. 2013a). In reaction mixture of PPO activity, 1 mL of 0.02 M citrate phosphate buffer, pH 6.0; proline (5 mg mL^{-1}), 0.5 mL; catechol (2 mg mL^{-1}), 0.5 mL; and 500 μL of appropriate diluted plant extract were used and read at 546 nm by time scan method for 3 min with 30-s intervals of readings. The mixture was aerated before addition of catechol which served as blank. One unit of enzyme is defined as the change in absorbance of 0.01 unit min^{-1}. In the reaction mixture of PO activity, 1 % (v/v) guaiacol in 0.01 M sodium phosphate buffer (pH 6.0) and 0.1 M H$_2$O$_2$ were added to the extract at the end to initiate the reaction followed by reading absorbance at 470 nm using time scan method for 3 min with 30-s intervals. One unit of enzyme is defined as the change in absorbance of 0.01 unit min^{-1} g^{-1} fresh weight of extract. Phenylalanine ammonia lyase (PAL) activity was estimated in root extracts (100 μL) using 2.5 mL of 0.2 %L-phenylalanine in 50 mM Tris-HCl (pH 5 to 6) buffer solution. The reaction mixture was incubated at 40 °C for 60 min. The amount of released trans-cinnamic acid from L-phenylalanine was measured at 290 nm. Distilled water instead of cyanobacterial cultures served as blank, and the details are given in Prasanna et al. (2013a). One unit of enzyme is defined as change in absorbance min^{-1} g^{-1} fresh tissue weight.

Chitosanase, endoglucanase (β-1,3-glucanase) and carboxy methyl cellulase (CMCase/β-1,4-endoglucanase) activities were assayed spectrophotometrically using glycol chitosan, laminarin and carboxy methyl cellulose respectively, as substrate using the methodology optimised earlier (Prasanna et al. 2013a). One unit of endoglucanase, CMCase and chitosanase activities was defined as 1 μmol of glucose and glucosamine (chitosanase) released per minute per millilitre culture filtrate, under the assay conditions respectively.

DNA fingerprinting and analyses

Anabaena filaments (approximately 50-mg wet weight) were collected by centrifugation and washed four times with sterile Milli-Q water. The samples were vortexed and homogenised using mini pestles, in order to rupture the filaments. The pellet was resuspended in 5 μL of Milli-Q water and used directly as a template in PCR reaction mixture for HIP-TG pattern analysis (Nayak et al. 2009). Amplification of HIP-TG sequence was done by using the following primer sequence (5′- to 3′-GCGATCACTG) in PCR reaction mixture. The primers were synthesized by Bangalore Genei Merck (India). The final concentration of constituents in PCR reaction mixture was 50 pmol of each primer, 2 mM dNTP, 2.5 % (v/w) BSA (bovine serum albumin), 10 % (v/v) DMSO (dimethyl sulphoxide), 1 U of *Taq* DNA polymerase, *Taq* buffer 2 μL and 5 μL of cyanobacterial filaments. The amplification was carried out for 35 cycles in a PEQLAB QB96 thermocycler following the protocol as pre-denaturation at 95 °C for 6 min, denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 65 °C for 5 min, and final extension at 65 °C for 16 min. After amplification, the products were analysed by running in 1.5 % agarose gel prepared using TAE (1×) buffer

at 80 V for 2 h. The PCR amplification for HIP-TG was repeated at least three times for each to ascertain the reproducibility of the band patterns. The size of bands in HIP-TG profiles were measured with the help of AlphaImager software by running 1-kb DNA ladder obtained from Bangalore Genei Merck (India).

DNA extraction, sequencing of 16S rDNA gene and phylogenetic analysis

DNA from plant roots of each treatment was extracted by using UltraClean plant DNA isolation kit (MoBio Inc., USA). Amplification of 16S rDNA gene was performed using primers fD1 and rD1 to isolate the nearly complete 16S rDNA gene sequence (Weisburg et al. 1991). The reaction mixture contained 1× *Taq* buffer, 2.5 mM $MgCl_2$, 0.3 mM each of the deoxynucleotide triphosphate, 10 pmol of each primer, 1 U *Taq* DNA polymerase, 50 ng of template DNA and Milli-Q to give final volume of 20 μL. The reaction mixture was incubated in a PEQLAB Primus 96 thermal cycler using the optimised program as described by Hisbergues et al. (2003), with annealing temperature of 50 °C. All PCR-related chemicals were purchased from Bangalore Genei Merck Pvt. Ltd., India. PCR-amplified products were purified and sequenced using both fD1 and rD1 primers for forward and reverse reactions, respectively. Sequencing of amplified gene was carried out by using Applied Biosystems ABI prism automated DNA sequencer (Applied Biosystems, USA). The 16S ribosomal RNA (rRNA) gene sequences were compared with sequences available in the NCBI database. Isolates were identified to species level on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in GenBank. Sequence alignment and comparison used the multiple sequence alignment tool CLUSTALW (Thompson et al. 2002) with default parameters. The phylogenetic tree was constructed on aligned data sets using the neighbour-joining (NJ) method (Saitou and Nei 1987) and the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1,000 random samples taken from multiple alignments (Felsenstein 1981).

Statistical analysis

The experiment was based on a completely randomised design (CRD), which included seven treatments, along with control. The data obtained in triplicates for the various parameters were analysed by ANOVA (analysis of variance) using MSTAT-C statistical package, and critical differences values were calculated at probability level of 0.05 %. Standard deviations (SD) are denoted as error bars in graphs. Correlations were analysed using Pearson's coefficient in the Microsoft Excel package. Path coefficient analyses were carried out by using Windostat version 8.5 and the procedure outlined by Singh and Chaudhary (1979).

Results

Growth parameters and nitrogen fixation

In the present investigation, all the cyanobacterial strains were able to form a close association with wheat roots in hydroponics experiment. The colonisation by these cyanobacteria was reflected by the presence of blue-green colonies on roots, which were visible to the naked eye and could not be dislodged even after gentle washing using distilled water. In order to study the degree of colonisation by cyanobacteria, the chlorophyll content of media, roots and shoots (Fig. 1 and Supplementary Table 1) was estimated, which revealed a significant increase in the values of media and plants in cyanobacteria-inoculated treatments. The chlorophyll values ranged from 0.065 to 2.904 mg mL^{-1} in the medium, and the highest value was recorded in treatment involving inoculation of *A. oscillarioides* CW3, followed by treatments *A. azollae* CW2 and *A. laxa* CW1 with values of 1.91 and 0.68 mg mL^{-1} chlorophyll. A significant increase of 80 to 90 % in chlorophyll accumulation in medium (Fig. 1a) over the control was recorded. Root chlorophyll values were highest in the treatment involving *A. laxa* RPAN8 with a value of 0.094 mg g^{-1} fresh weight, followed by treatment with *A. oscillarioides* CW3 and *A. azollae* CW2 with values of 0.085 and 0.045 mg g^{-1}. The treatment involving RPAN8 *A. laxa* and *A. oscillarioides* CW3 showed the highest root chlorophyll, with an increase of 20 to 80 % over control (Fig. 1b). An increase of 28 to 70 % in shoot chlorophyll, as compared to control, was observed, with the treatment involving inoculation of *A. laxa* RPAN8 recording the highest increase in chlorophyll (an index of photosynthesis). Shoot chlorophyll ranged from 0.30 to 1.07 mg g^{-1}; the highest chlorophyll was recorded in treatment involving inoculation of *A. laxa* RPAN8 with values of 1.075 mg g^{-1}, followed by treatment with *Calothrix* sp. and *A. oscillarioides* CW3.

The ARA values were significantly higher (20-fold increase over uninoculated control) in treatment involving inoculation of *A. laxa* RPAN8, with a value of 2.34 μmol C_2H_4 $plant^{-1}$ h^{-1} (Table 1). Nitrogen-fixing potential was recorded for whole plants which revealed values ranging from 0.11 in control plants (uninoculated) to 2.34 μmol ethylene $plant^{-1}$ h^{-1} (Fig. 1c), equivalent to 7 nmoles N $plant^{-1}$ h^{-1} (obtained by converting these values in terms of N fixed, using a factor of 3, as suggested by Dommergues et al. (1973)). The treatments involving inoculation of *Calothrix* sp. and *A. oscillarioides* C20 exhibited at par values of 1.75 and 1.48 μmol C_2H_4 $plant^{-1}$ h^{-1} respectively. ARA showed a

Fig. 1 Influence of
cyanobacterial inoculation on
chlorophyll content after 14 days
of growth. **a** Percent change in
chlorophyll content in root and
medium over control; **b** percent
change in chlorophyll of shoot
and medium over control; **c**
Nitrogen-fixing potential of the
associations measured as
acetylene-reducing activity
(ARA) and expressed in terms of
nanomoles of N fixed per plant
per hour
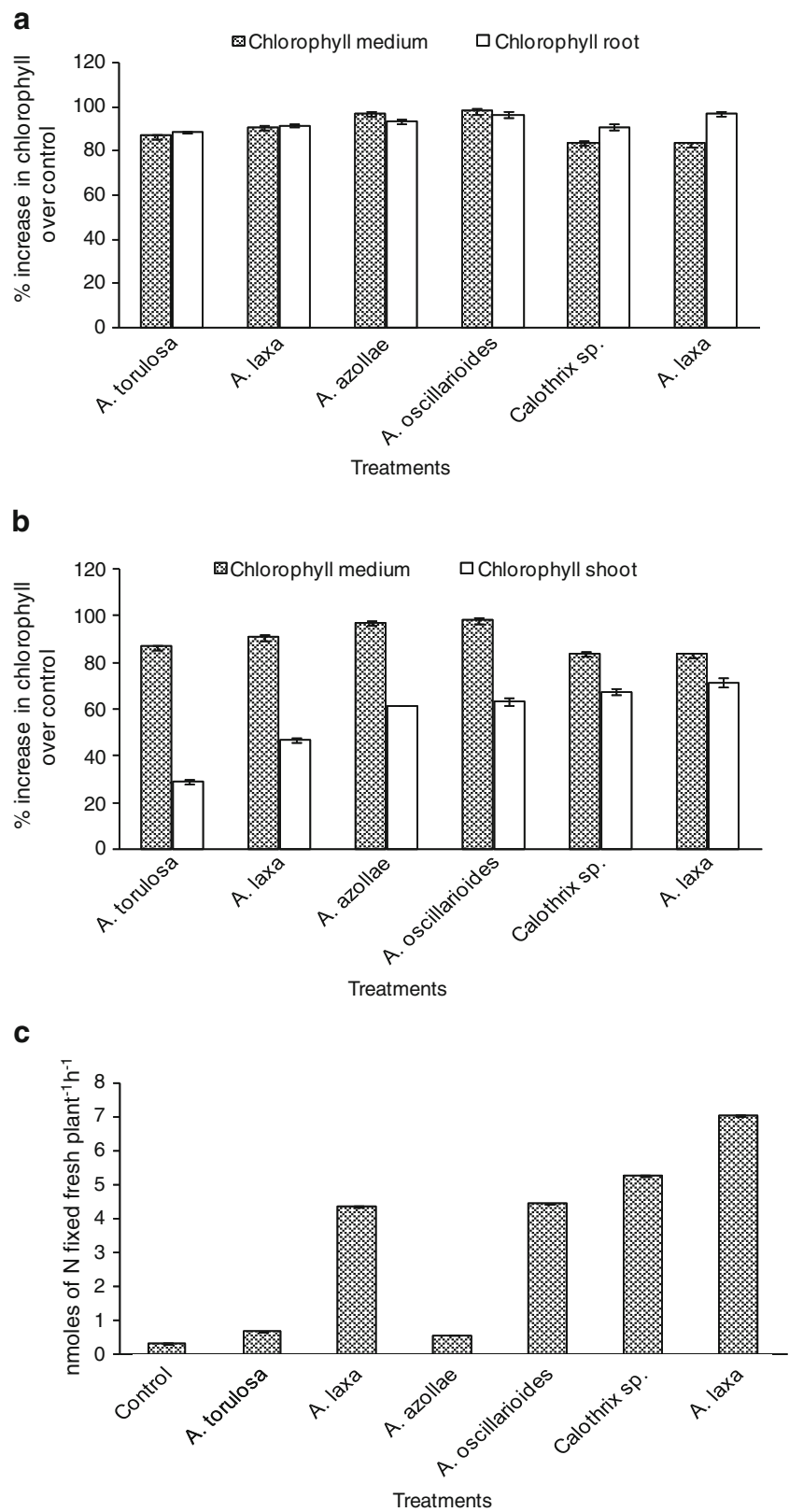
positive correlation with root chlorophyll ($r=0.70$) and shoot chlorophyll ($r=0.75$).

The fresh weight of plants measured after 2 weeks (Table 1) revealed an increase of 35–60 %, as compared to that in

Table 1 Effect of inoculation on the metabolic activity of wheat roots

Treatments	Plant fresh weight (g)	ARA (nmoles ethylene plant^{-1} h^{-1})	CMC activity (β 1,4-glucanase, IU g^{-1} fresh weight)	Chitosanase activity (IU g^{-1} fresh weight)	PPO activity (IU g^{-1} fresh weight)	PAL activity (IU g^{-1} fresh weight)
Control	0.210±0.02	0.11±0.01	12.2±0.04	0.066±0.012	8.00±0.00	80.99±1.80
Anabaena torulosa BF1	0.328±0.03	0.23±0.02	14.4±0.04	0.058±0.007	20.00±2.51	106.79±2.75
Anabaena laxa CW1	0.443±0.03	1.46±0.14	15.0±0.13	0.103±0.001	20.00±4.00	106.19±3.60
Anabaena azollae CW2	0.418±0.03	0.19±0.03	12.6±0.88	0.086±0.006	22.67±4.62	124.79±4.53
Anabaena oscillarioides CW3	0.505±0.01	1.48±0.04	16.4±0.57	0.063±0.007	45.33±2.31	143.99±8.25
Calothrix sp. RPC1	0.514±0.01	1.75±0.12	17.0±0.37	0.063±0.013	66.67±6.11	157.19±3.75
Anabaena laxa RPAN8	0.516±0.03	2.34±0.07	16.4±0.57	0.058±0.006	66.67±4.62	172.19±2.75
SEM	0.01	0.05	0.18	0.03	2.55	3.03
CD	0.30	0.14	0.51	0.08	7.01	8.36

All values represent means ± SD; $n=3$

ARA acetylene-reducing activity, *CMC* carboxy methyl cellulose , *PPO* polyphenol oxidase, *PAL* phenylalanine ammonia lyase

control. The fresh weight values ranged from 0.21 to 0.51 g fresh weight, with the highest plant fresh weight recorded in treatment involving inoculation of *A. laxa* RPAN8 followed by treatment *Calothrix* sp. and *A. oscillarioides* CW3. A similar trend of 15–40 % increase in plant dry weight (Supplementary Table 1) was also recorded with respect to control.

Activity of hydrolytic and plant defense enzymes

The CMCase (β-1,4-endoglucanase) activity (Table 1) in root tissues was very high, as compared to the activity of other enzymes, ranging from 12.20 IU g^{-1} fresh weight (in uninoculated control) to 17 IU g^{-1} fresh weight in treatment involving inoculation of *Calothrix* sp. CMCase activity showed a significant positive correlation with endoglucanase ($r=0.87$), shoot chlorophyll ($r=0.70$), PPO ($r=0.92$), ARA ($r=0.88$), and plant fresh weight ($r=0.83$). CMCase activity recorded the highest percent increase in T6 (28 %), T5 (25 %) and T7 (25 %) over control. β-1,3-endoglucanase showed a significant enhancement of activity in the inoculated treatments— *A. torulosa* BF1 and *A. laxa* RPAN8—as compared to that in the control. β-1,3 endoglucanase activity (Supplementary Table 1 and Fig. 2a) in root tissues ranged from 0.001 to 0.056 IU g^{-1} fresh weight, with the highest endoglucanase activity being recorded in treatment involving inoculation of *Calothrix* sp. with a value of 0.056 IU g^{-1} fresh weight. This was followed by *A. oscillarioides* CW3 and *A. laxa* RP8 recording 0.043 and 0.038 IU g^{-1} fresh weight, which were statically at par with control. A significant (25 to 28 %) increase in CMCase activity of treatment involving *A. oscillarioides* CW3, *Calothrix* sp. and *A. laxa* RPAN8 was recorded as compared to that in control. Chitosanase activity ranged from 0.066 to 0.103 IU g^{-1} fresh weight. The highest activity was recorded in treatment involving inoculation of *A. laxa* CW1, followed by treatment with *A. azollae*

CW2 and uninoculated control (Table 1). A positive correlation of CMCase ($r=0.88$), β-1,3-endoglucanase ($r=0.78$) and PPO ($r=0.88$) with ARA was recorded. A positive correlation ($r=0.78$) was observed between β-1,3-endoglucanase activity and ARA.

Plant defense enzyme activity, in terms of peroxidase (Table 1), polyphenol oxidase and phenylalanine ammonia lyase enzyme (Supplementary Table 1), was analysed in the root samples of the wheat seedlings. Peroxidase activity in root samples ranged from 29.00 to 141.00 IU g^{-1} fresh weight (Fig. 2b). The highest peroxidase activity of 141 IU g^{-1} fresh weight was recorded in treatment involving inoculation of *A. laxa* RPAN8, followed by *Calothrix* sp. and *Anabaena* sp. CW2 with values of 123 and 117 IU g^{-1} fresh weight. However, these values were statistically at par with control (29 IU g^{-1} fresh weight). Interestingly, a significant positive correlation of ($r=0.44$) with chitosanase, ARA ($r=0.88$), plant fresh weight ($r=0.75$), PAL ($r=0.81$), activity of CMCase ($r=0.92$) and endoglucanase ($r=0.91$), as also with root chlorophyll ($r=0.61$) and shoot chlorophyll ($r=0.78$) were also recorded. PPO activity in roots ranged from 8 to 66.66 IU g^{-1} fresh weight. The treatment involving inoculation of *A. laxa* RPAN8 showed the highest value of 66.66 IU g^{-1} fresh weight followed by *Calothrix* sp. and *A. oscillarioides,* with values of 66.65 and 45.33 IU g^{-1} fresh weight, which were statistically at par with control. A significant increase of 60 to 88 % in PPO activity was recorded in these treatments as compared to that in control. Phenylalanine ammonia lyase activity in root ranged from 80.99 to 172.18 IU g^{-1} fresh weight. The highest activity was recorded in microbial inoculation involving *A. laxa* RPAN8 with a value of 172.18 IU g^{-1} fresh weight, followed by treatment with *Calothrix* sp. and *A. oscillarioides*. The observations on *A. laxa* (CW1) and *A. azollae* (CW2) were also found to be statistically at par with those in the uninoculated control

Fig. 2 Modulation of activity of plant hydrolytic, defence enzyme and plant dry weight by cyanobacterial inoculation. **a** Percent change in endoglucanase activity over control; **b** percent change in peroxidase activity over control; **c** percent change in plant dry weight over control
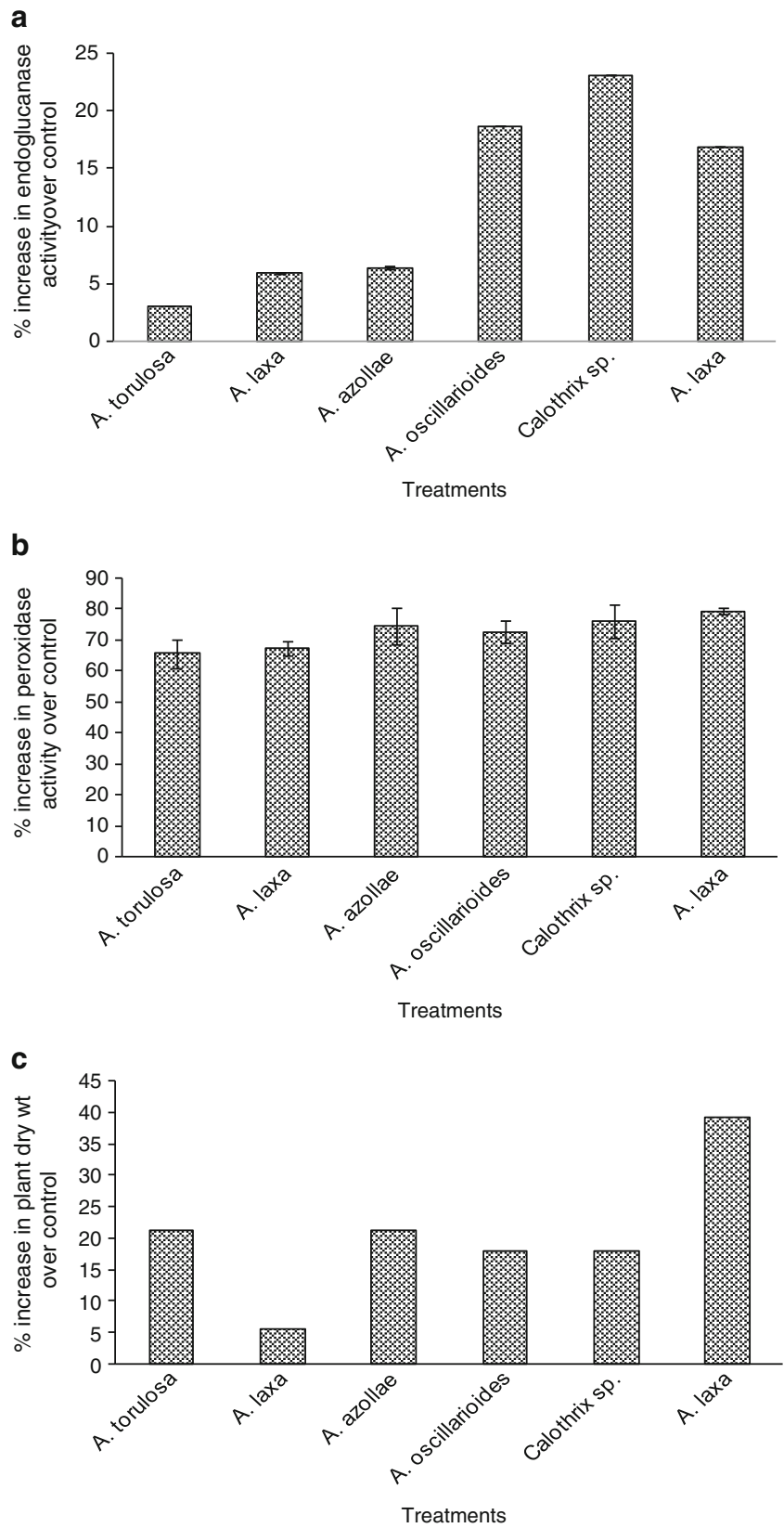
(80.99 IU g^{-1} fresh weight). PAL shows a significant positive correlation ($r=0.81$) with peroxidase; significant 52.9 % increase in PAL activity was recorded in T6 (*Calothrix* sp.) as compared to that in control. The plant dry weight values ranged from 0.06

to 0.10 g dry weight, and the highest values were recorded in *A.-laxa*-RPAN8-inoculated treatment (Fig. 2c).

PCR-based DNA fingerprinting and their 16S-rDNA-based phylogenetic analyses

Comparison of DNA fingerprints of wheat root DNA with those of the treatments involving inoculation with cyanobacterial strains (Fig. 3) showed that the common bands were in the range of 300–4,000 bp, with the maximum number of bands in the 500 to 3,500 bp region. The DNA fingerprints of rice roots of control (uninoculated) treatment seedlings did not show any bands. The treatment inoculated with *A. torulosa* BF1 showed a distinct band pattern, in which four unique bands were observed of sizes 2,690 and 1,523 bp and duplet of 1,016 and 824 bp, which were common to both pure cultures and treatment. In the sample from *A. laxa* (CW1), one band of 1,372 bp was common. The profile of treatment with *A. azollae*(CW2) revealed a pattern comprising a triplet of 3,854, 3,570 and 2,944 bp and duplet of 1,513 and 1,372 bp, which were similar to that of pure culture used for inoculation. *A.-oscillarioides*-inoculated treatment showed a band pattern of 1,372, 1,229, 604 and 464, which matched with the fingerprint of the pure culture. T6 showed a band pattern, made up of bands of 3,272, 2,831, 1,478 and 1,280 bp, identical with that of the inoculated pure culture fingerprint. *A. laxa* (RPAN8) showed only one band of 2,005 bp, also present in the DNA profile of the pure culture. The details of the common bands present in both root DNAs of all the treatments vis-a-vis pure cultures (Supplementary Table 2).

The 16S rDNA sequences generated from plant root DNA of all the samples were compared with those of the inoculated cyanobacterial strains (available in NCBI database), and a phylogram was generated, which revealed two major clusters

(Fig. 4). Both clusters were 83 % similar. The first cluster comprised two subclusters, in which *A. laxa* CW1 showed 100 % similarity with the sequence of *A. laxa* RPAN8, available in the database. The treatment involving inoculation of *A. azollae*(CW2) showed the nearest similarity with *Anabaena flos-aquae* and 99 % similar to *A. laxa* (CW1) and the sequence of RPAN18 *A. laxa* available in the database. In the second subcluster, *A. oscillarioides* showed 99 % similarity with the NCBI sequence of RPAN69 *A. oscillarioides*. *A. laxa* RPAN8 showed 100 % similarity with that of *Anabaena variabilis* (AB016520), while *A. torulosa* BF1 and NCBI sequence GU396091 (*A. torulosa* BF1) exhibited 100 % similarity. The treatment inoculated with *Calothrix* sp. showed 100 % similarity with the deposited sequence of this strain in the NCBI.

Path coefficient analyses

The influence of ARA and fresh weight with other parameters was selected for path coefficient analyses, as a significant effect was observed. Positive direct effects of chlorophyll content in roots (0.91) and PPO (0.762) besides plant fresh weight (0.58) and endoglucanase activity (0.46) on ARA were recorded (Table 2 and Fig. 5). The correlation between the activity of PPO, CMCase, PAL and endoglucanase with ARA was very high (0.77–0.85). The residual effect was 0.148, indicative that all the parameters taken into consideration played a major role (84.2 % or 0.842) in the enhancement of ARA, brought about by cyanobacterial inoculation. The fresh weight of plants was positively correlated (0.7–0.8) with all the parameters, except for a negative correlation with endoglucanase activity. The positive direct effect of PAL (0.86) on fresh weight of plants was very high, followed by the effect of chlorophyll content of growth medium, CMCase
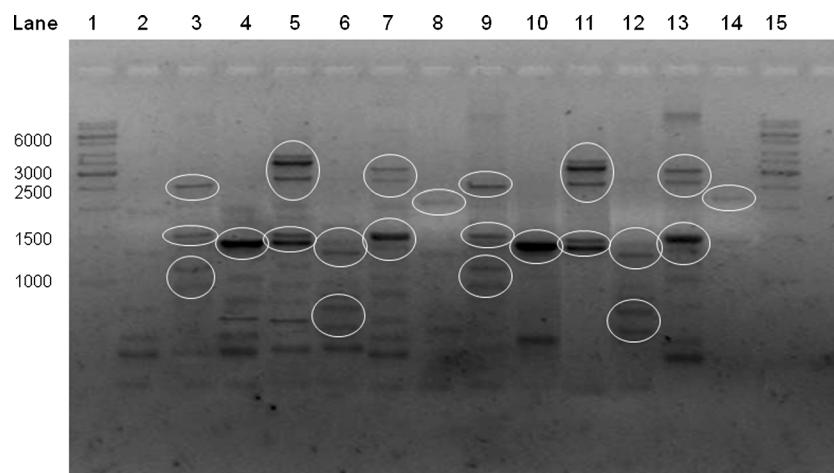

Fig. 3 HIP-TG PCR-based DNA fingerprints of wheat plant roots and pure cyanobacterial samples. *Lane 1* is the 1-kb marker; *lanes 2–8* are root samples from uninoculated control, *Anabaena torulosa* BF1 inoculated, *Anabaena laxa* CW1 inoculated, *Anabaena azollae* CW2 inoculated, *Anabaena oscillarioides* CW3 inoculated, *Calothrix* sp. RPC1 inoculated, and *A. laxa* RPAN8 inoculated. *Lanes 9–14* are pure culture samples of *A. torulosa* BF1, *A. laxa* CW1, *A. azollae* CW2, *A. oscillarioides* CW3, *Calothrix* sp.RPC1, and *A. laxa* RPAN8
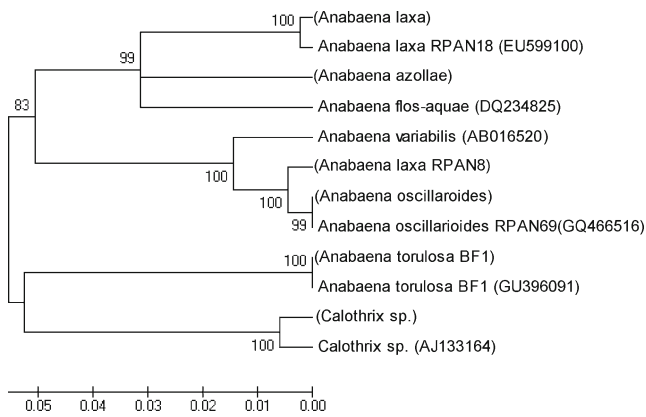
Fig. 4 Phylogram based on 16S rDNA sequences generated using PCR-based amplification of plant root DNA from the treatments and reference sequences retrieved from NCBI database

and ARA on fresh weight. The residual effect was 0.145, which shows that the increase in fresh weight is due to the attributes investigated as influenced by cyanobacterial inoculation (Table 2).

Discussion

Cyanobacteria represent a highly heterogenous assemblage of photosynthetic prokaryotes, which are employed as inoculants for improving crop productivity and soil health in several crops, including rice, wheat, legumes, fruits and vegetables (Venkataraman 1972; Kaushik 2004; Nayak et al. 2004; Karthikeyan et al. 2007; Prasanna et al. 2008, 2009, 2013b, c; Nain et al. 2010;). They are known to produce phytohormones, provide fixed nitrogen and make available other nutrients to plants. Reports are also available on their potential as bio-control agents, especially against soil-borne phytopathogenic fungi (Manjunath et al. 2009; Chaudhary et al. 2012; Prasanna et al. 2013a). In order to increase the benefits of cyanobacterial nitrogen fixation or its plant-growth-promoting properties, there is a need to develop tighter associations,

especially as alternatives to obviate repeated inoculation or genetic engineering to mediate transfer of *nif* genes into plants. The present study was undertaken to evaluate a set of cyanobacterial strains, in terms of their nutritional and biochemical interactions with wheat roots, as a prelude to developing effective associations for use in integrated nutrient management practices in wheat.

In the present investigation, the cyanobacterial strains formed a close association and enhanced the chlorophyll content of plants and fresh/dry weight. A similar observation was recorded earlier for a different set of strains by (Gantar et al. 1991). Some of these cyanobacterial isolates (originally from wheat rhizosphere) used in this investigation are known to produce growth-promoting substances and remain in association with roots, both extra- and intra-cellularly (Jaiswal et al. 2008; Karthikeyan et al. 2007, 2009; Prasanna et al. 2008, 2013a), but the mode of entry or biochemical changes mediating the colonisation could not be elucidated. Cultivation of wheat with cyanobacteria was found to increase root dry weight and chlorophyll (Obreht et al. 1993), as observed in the present investigation too. Prasanna et al. (2013c) recorded improvement in fresh and dry biomass of plants by 12–25 %, through the use of cyanobacterial formulations. The strains used in this study are known to produce IAA (Prasanna et al. 2008), which may be responsible for the increase in plant biomass and chlorophyll. Despite the availability of published research in related areas, the exact mechanisms involved and employed by cyanobacterial cells to enter the plant is not clear; root hair exudates seem to attract the filaments and may be involved in recognition of some lectins (McCowen et al. 1986; Knight and Adams 1996).

Anabaena or *Nostoc* strains are known to colonise the roots of wheat and exhibit associative nitrogen fixation (Gantar et al. 1991, 1995; Spiller et al. 1993; Gantar and Elhai 1999; Gantar 2000). The cyanobacterial strains in the present study also enhanced plant-growth-promoting traits such as N-fixing potential, measured using the acetylene-reducing assay/ARA. The positive correlation of ARA with root chlorophyll ($r=0.70$)

Table 2 Correlation analyses and direct and residual effects of factors influencing ARA and fresh weight in wheat

Pathway of association	CMC	Chitosanase	Endoglucanase	Media chlorophyll	Root chlorophyll	Shoot chlorophyll	PO	PPO	PAL	ARA
Direct effect with ARA	0.028	0.149**	0.460***	−0.606	0.913***	0.180**	−0.095	0.762***	−1.461	0.580***
Correlation with ARA	0.872***	−0.167	0.777***	−0.002	0.659**	0.745***	0.658**	0.846***	0.800***	0.809***
Residual effect	0.148									
Direct effect with fresh weight	0.332***	0.095	-0.59	0.59***	−0.61	−0.03	0.20**	0.05	0.85***	0.55***
Correlation with fresh weight	0.800***	−0.008	0.795***	0.395	0.735***	0.842***	0.884***	0.816***	0.876***	0.809***
Residual effect	0.145									

ARA acetylene-reducing activity, *CMC* carboxy methyl cellulose , *PPO* polyphenol oxidase, *PAL* phenylalanine ammonia lyase, *PO* peroxidase

*significant at $P \leq 0.1$, **significant at $P \leq 0.05$, ***significant at $P \leq 0.01$ respectively
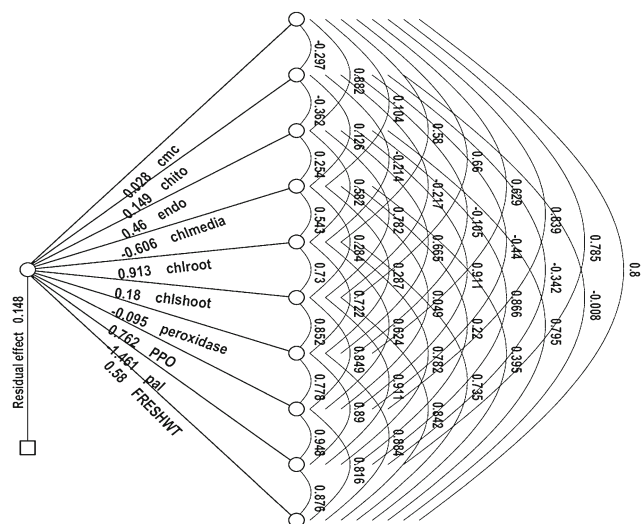
Fresh weight
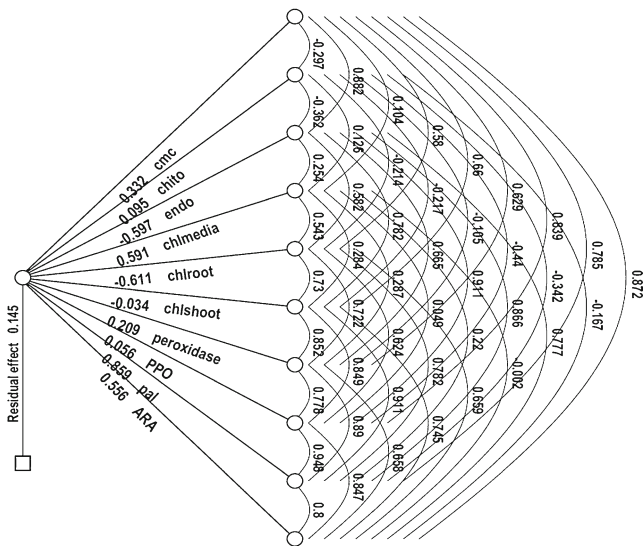
ARA

Fig. 5 Path diagram representing the relationships of plant fresh weight and ARA activity with the other parameters

and shoot chlorophyll ($r=0.75$) revealed that the increase in ARA is growth-linked and indirectly colonisation, as has been observed in other studies (Gantar et al. 1991, 1995; Obreht et al. 1993). This shows the synergistic effect of association of cyanobacteria with wheat, which leads to improved associative N fixation. Path coefficient analysis for the ARA and fresh weight with other parameters was undertaken to partition the correlations into direct and indirect effects. The analyses revealed the parameters taken into consideration, including the activity of defense, and hydrolytic enzymes were significant in their role in modulating ARA and fresh weight.

The regular use of agricultural land for the cultivation of major crops has led to the gradual exhaustion of minerals and nutrient imbalances, emphasizing an urgent need for employing

multifunctional microbial consortia/bio-fertilisers which can promote the growth of plants by producing auxins/bioactive molecules and helping to sustain the soil fertility. The role of plant-growth-promoting rhizobacteria (PGPR) in enhancing plant biometric parameters/yield and biological control of soil-borne pathogens has been intensively investigated by several workers (Mäder et al. 2011; Piromyou et al. 2011). Cyanobacteria are known to produce antibacterial and antifungal metabolites/enzymes which help in controlling diseases, besides secreting mucilage and polysaccharides which can improve soil structure and porosity (Prasanna et al. 2008; Gupta et al. 2013). The plant cell wall is a complex mixture of polysaccharides, proteins and other defense compounds, which hinder the invasion of any foreign organism. One of the predicted routes of bacterial penetration into plant tissue besides mechanical/physical modes is through the secretion of an extensive repertoire of glycoside hydrolases, lyases and esterases which can target the numerous linkages as a means of cell wall degradation, especially for pathogen. In the present investigation, the cyanobacterial strains used are known to exhibit hydrolytic enzyme activity such as glucanases or chitosanases (Prasanna et al. 2009; Gupta et al. 2012). The major role of β-1,4-endoglucanases in plants is to degrade the cell wall, but this enzymatic activity can also contribute significantly in the biosynthesis of cellulose, polysaccharide modifications and loosening of cell wall during elongation of cells. Therefore, such type of enzymatic activity has been reported during normal growth and development, as well as in growing shoot, root and germination of grains (Buchanan et al. 2012). The enhanced activity of cell-wall-hydrolysing enzymes in the root tissues of inoculated plants, with increase of 3.1–18.7 % over uninoculated control, can be attributed to the stimulation of the plant-related enzymes, as a result of the cyanobacterial inoculation. The positive correlation ($r=0.78$) observed between β-1,3-endoglucanase activity and ARA is in congruence with the earlier reports of Kovtunovych et al. (1999) using *Klebsiella* spp., who reported an increase in pectate lyase activity (another cell-wall-hydrolysing enzyme) showing positive correlation and concomitant with a tenfold higher rate of internal colonisation of wheat roots and higher ARA. It needs to be emphasized here that despite the enhanced values of these enzymes, they are not potent enough for complete plant cell wall degradation.

The inoculated cyanobacteria also elicited pathogenesis-related (PR) proteins, which serve as plant protection strategies against pathogens and pests. The phenomenon of induced systemic resistance (ISR) and systemic acquired resistance (SAR) is well investigated in terms of bio-control mediated by endophytic bacteria or plant-associated bacteria (Loon et al. 1998; Kloepper and Ryu 2006), but very few reports exist on cyanobacteria (Prasanna et al. 2013a; Singh et al. 2011). A differential systemic accumulation of phenylpropanoids in plant leaves, when inoculated with different cyanobacterial strains, emphasizes that cyanobacterial

inoculation correlates positively with plant growth promotion and stress tolerance in rice (Singh et al. 2011). These findings support the conclusions of this study on the significant role of defense/pathogenesis enzymes not only in improving colonisation but also in enhancing plant fitness. Several workers (Karthikeyan et al. 2006; Patil et al. 2011) observed that Actinomycetes help in protecting tomato plants against *Rhizoctonia solani*, and such plants showed better growth in terms of high chlorophyll and disease reduction. In an earlier study, Prasanna et al. (2013a) investigated the cyanobacteria—phytopathogenic fungi—tomato plant interactions, as a prelude towards developing suitable biological options for combating biotic stress (*Fusarium* wilt) and enhancing plant vigour.

Cyanobacteria have been mainly identified with the help of morphological characters like shape, size of cells, place and position of heterocyst, or nature of growth in liquid/agar media (Nayak et al. 2009). These prokaryotes have highly conserved repetitive sequences in their genome such as highly iterated palindrome (HIP-TG) sequences, which are specific to these organisms. Such sequences can be an important tool for DNA fingerprinting, besides helping in studying the presence and association with their hosts (Rasmussen and Svenning 1998; Rai et al. 2000; Prasanna et al. 2012). In order to study the colonisation and association of cyanobacterial strains, analyses of the PCR-based profiles of wheat root DNA using repeat sequences were undertaken. DNA fingerprints from the samples revealed that specific bands could be distinguished for monitoring the presence of the inoculated strain and can be valuable for confirming colonisation. Earlier reports on evaluating the role of cyanobacteria on soil microbiological and plant parameters employing agronomic and molecular tools (Singh et al. 2011; Prasanna et al. 2013b) further emphasize the significance of our findings.

In the present investigation, path coefficient analyses helped to correlate and prove the significance of the parameters evaluated in this study, towards the enhancement in plant vigour (using fresh weight and ARA as indices). In general, path coefficient analysis has been employed mainly in plant breeding or selection of varieties or yield analyses, but is not used for analyses of microbial inoculation, as evidenced from published literature. The observations from the analyses in this study serve to highlight the significant role of activity of hydrolytic and defense enzymes in enhancing growth, ARA and thereby colonisation of cyanobacteria in wheat roots.

This study is a novel report which illustrates the mode of entry and colonisation in wheat plants by cyanobacteria through stimulating the activity of plant defense and pathogenesis enzymes and is further confirmed using DNA-based profiles. The colonisation by cyanobacteria led to improved plant growth, nitrogen fixation and nutrient mobilisation in such associations. This can also be considered a step forward in the dream of developing nitrogen-independent cereals, without the need for genetic engineering and prove more time and cost effective. Such plants need to be tested under different agro-climatic conditions to evaluate their agronomic efficiency.

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