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Full Length Research Paper

Screening of native plant growth promoting cyanobacteria and their impact on *Triticum aestivum* var. Uqab 2000 growth

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In the current study cyanobacterial strains isolated from rice fields were evaluated as biofertilizers. They were tested for different plant growth promoting traits such as phosphate solubilization, nitrogen fixation and, hydrogen cyanide and auxin production. The two selected cyanobacterial strains were identified as *phormidium* SM-14 and SM-15. Both strains were able to solubilize phosphate, fix atmospheric nitrogen and produce hydrogen cyanide. Cyanobacteria produced variable amount of auxin in the presence of different concentrations of L-Tryptophan. Cyanobacterial strains used as biofertilizers have tremendous potential to enhance growth of *Triticum aestivum* var. Uqab 2000 in control conditions.

Key words: Cyanobacteria, wheat, auxin, BG11 medium, *Phormidium*.

INTRODUCTION

The use of chemical fertilizers in agriculture has been enormously amplified due to increased demand of food and there is little doubt regarding the use of chemical fertilizers coupled with crops to achieve significant increment in yield. However, their indiscriminate use has also resulted in serious consequences for both soil and water environments. Cyanobacteria currently seem to be offering a potentially environmental friendly alternative to the use of chemical fertilizers (Vaishampayan et al., 2001; Sinha et al., 2002; Choudhury and Kennedy 2005; Rai, 2006). Cyanobacteria are photosynthetic prokaryotes and colonizing microorganisms that are found throughout the world and they are exceptionally well adapted to a wide array of environmental conditions (Paerl et al., 2000; Karthikeyan et al., 2008; Kirlwood et al., 2008). They can enhance the plant growth directly and/or indirectly. The direct ways include the production of various plant growth promoting biologically active substances including phytohormones, such as auxin (Sergeeva et al., 2002; Prasanna et al., 2010), gibberellins (Rodriguez et al., 2006) and cytokinins (Stirk et al., 2002; Hussain and Hasnain, 2009). The indirect promotion of plant growth occurs when cyanobacteria prevent or counter deleterious effects of one or more phytopathogenic microorganisms (Moussa and Shanab, 2001; Rizk, 2006; Kim, 2006; Abo-Shady et al., 2007; Tassara et al., 2008; Kim and Kim, 2008). Cyanobacteria are also capable of fixing atmospheric nitrogen (Choudhury and Kennedy, 2004; Osman et al., 2010). Several studies have reported that cyanobacteria can improve the plant growth by improving the soil structure as they have potential to secrete extracellular polysaccharides that help in soil aggregation and water retention (Hill et al., 1994; Mazor et al., 1996; Maqubela et al., 2009). In addition, the use of cyanobacteria can increase the carbon and nitrogen status of soil (De Caire et al., 2000; De Cano et al., 2002; Azia and Hashem, 2004; Pandey et al., 2005; Malam Issa et al., 2007; Obana et al., 2007; Nisha et al., 2007; Swarnalakshmi et al., 2007; Maqubela et al., 2009; Saadatnia and Riahi, 2009; Maqubela et al., 2009). Keeping all these attributes in mind the present study was designed to isolate, identify and screen native plant growth promoting cyanobacteria. The effects of these locally isolated

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cyanobacteria on the growth of wheat were also demonstrated.

MATERIALS AND METHODS

Isolation, purification and characterization

Soil sample was collected and transferred under aseptic conditions to the laboratory and stored in an ice pack at 4°C in the laboratory. One milliliter of the appropriate (10^{-5} to 10^{-7}) dilutions of the soil samples was plated on BG11 medium (Rippka et al., 1979) containing cyclohexamide (100 µg/ml) for the isolation of *Phormidium* sp. Filaments were picked by using a sterile inoculating needle and streaked to a new BG11 plate incubated at 25 ± 2°C, illuminated by 18 µmol photons m^{-2} s^{-1} light. This process was repeated three to four times until axenic culture was obtained. Contamination and cyanobacterial growth was monitored on weekly basis under a dissecting microscope. The axenic condition of the cultures was tested by transferring pieces of agarose block with the trichomes to LB agarose medium plates. Finally purified filaments were transferred to liquid medium using sterile inoculating needle. After purification the strains were characterized morphologically and physiologically.

Strains identification

Cyanobacterial strains were identified by 16S rDNA sequencing. 15 days old cyanobacterial cultures were harvested by centrifugation at 25000 g for 10 min. Then cultures were suspended in 500 µl wash buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA and 50 mM NaCl) separately and homogenized using a Mixer Mill MM 300 (Qiagen) at 15 to 25 Hz for 10 min. The homogenate was centrifuged at 2500 g for 10 min and the supernatant containing the impurities (such as polysaccharides and humic acid) was decanted out. The pellets so obtained were again washed with the wash buffer mentioned above and pulled off in 500 µl of 10% SDS. Now 10 µl of 5 mg/ml of proteinase k was added to the pellet containing 10% SDS. These samples were incubated at 37°C for one hour in a water bath shaker. Following incubation, 500 µl pre-warmed extraction buffer containing 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2.5 M NaCl, 3% CTAB, and 1% (v/v) 2-mercaptoethanol was added and kept at 65°C for one hour in a water bath shaker. At this stage approximately 1 ml of suspension was obtained for each sample. These suspensions were allowed to cool down slowly to room temperature. Thereafter, 1 ml (equal to the volume of suspension obtained) of chloroform: isoamyl alcohol (24:1) solution was added. This gave rise to two distinct layers, an upper aqueous and a lower organic solvent layer in each tube. The upper aqueous transparent layers from each tube were transferred to another 10 ml tube. Care was taken to avoid any contamination from the lower layer. To the aqueous layer, 200 µl (1/10 of total volume) 3 M sodium acetate (pH 5.2) and 2 ml (double volume) of pre-chilled ethanol were added and the tubes left over night at −20°C for nucleic acid precipitation. These suspensions were centrifuged at 18000 g for 15 min to pellet the precipitated nucleic acid. The pellets so obtained were collected in 1.5 ml micro-centrifuge tubes and washed in 70% ethanol. The pellets were dried using a hot air blower for complete removal of ethanol. Care was taken to avoid over-drying of the pellets. These pellets were resuspended in 50 µl of TE buffer (Tris-HCl 10 mM pH 8.0 and EDTA 1 mM). Extracted DNA was amplified by using forward primer 27 F1 (5´-TAGTGTAAAACGGCCAGTAGAGTTTGATCCTGGCTCAG-3´) and reverse primer 409R (5´-TTACAACCCAAGGGCCTTCCTCCC-3´). The amplified and purified DNA was sequenced using Automated Sequencer (Applied Biosystem; Model 3100).

Plant growth promoting traits

Cyanobacterial strains were evaluated for different growth promoting traits such as phosphate solubilization, nitrogen fixation, hydrogen cyanide (HCN) and IAA production. Phosphate solubilization was determined qualitatively by streaking strains on BG 11 agar plates containing tricalcium phosphate (0.3%). Development of a clear zone around the growth of cyanobacteria was observed after incubation at 24±2°C for 15 days. Nitrogen fixation potential was examined by growing them in nitrogen free media (BG11 without $NaNO_3$). HCN production was determined as described by Ahmad et al. (2008). The Salkowski colorimetric technique was used for the estimation of auxin in cultures of cyanobacteria (Glickmann and Dessaux, 1995). The cyanobacteria were grown on BG 11 media, pH 7 at 25°C for 15 days supplemented with different concentrations (0, 100, 200, 300, 400, 500, 700, 900 and 1000 µg/ml) of filter sterilized tryptophan. After 15 days, cyanobacterial cells were removed by centrifugation and Salkowski reagent was added to the supernatant in a ratio of 2:1 (v/v). Concentrations of auxin-like substances were estimated by taking 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.

Plant growth experiments

Pot experiments in laboratory were conducted to evaluate the phytostimulatory effect of cyanobacterial strains on *Triticum aestivum*. Seeds of *T. aestivum* var. Uqab 2000 procured from NARC Islamabad, Pakistan, were surface-sterilized with 0.1% $HgCl_2$ for 5 to 7 min with continuous shaking. The seeds were then rinsed five times with sterile water to remove any trace of $HgCl_2$. Seeds were incubated in cyanobacterial suspension (filaments of 15 days old cultures were homogenized to distribute them equally in the suspension) adjusted to 1 µg/ml Chlh-a. Water treated seeds were used as control. The pots (12 × 12 cm) used in experiment were sterilized by dipping them in 5% sodium hypochlorite solution for 20 min. Control and inoculated seeds (8 seeds per pot) were sown in sterilized pot containing autoclaved soil (approximately 300 g). 192 pots were labeled properly. The pots were kept at 25 ± 2°C, 60% relative humidity, 12 h photoperiod, and the light intensity was adjusted to 180 to 200 µmol m^{-2} s^{-1} for 15 days. Pots were observed and watered regularly during this period. After 15 days, plants were harvested and their growth parameters were analyzed.

RESULTS AND DISCUSSION

Isolation, purification and characterization

The cyanobacterial strains were collected from rice field located in agricultural area of Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan. The filamentous strains were purified and characterized morphologically and physiologically as given in Table 1. For morphological characterization of the plant growth promoting cyanobacterial strains SM-14 and SM-15, cell color and width were recorded. Both the strains were dark blue green in color but had different size width given in Table 1. The impact of varying temperatures (20, 25, 30 and 35°C) and pH (5, 6, 7, 8 and 9) on the growth of the cyanobacterial strains was recorded. The optimum temperature and pH for the growth of both the strains

Table 1. Morphological characterization of *phormidium* strains.

Strain	Shape	Cell width (µm)	Color	Hormogia cell	Heterocyst	motility
SM-14	Filamentous	6-7	DG	+	-	+
SM-15	Filamentous	4-5	DG	+	-	+

Table 2. Strains of *Phormidium* with their accession number identified by 16S rRNA gene sequencing isolated from *Oryza sativa* filed.

Strain	Identified as	Sequence length	Maximum homology (%)	Accession number
SM-14	*Phormidium*	1020	99	JF70 3677
SM-15	*Phormidium*	1246	100	JF703678

Table 3. Growth promoting traits of *Phormidium* strains.

Isolates	Auxin production	Nitrogen fixation	Phosphate solubilization	Hydrogen cyanide production
Smz-14	+	+	+	+
Smz-15	+	+	+	+

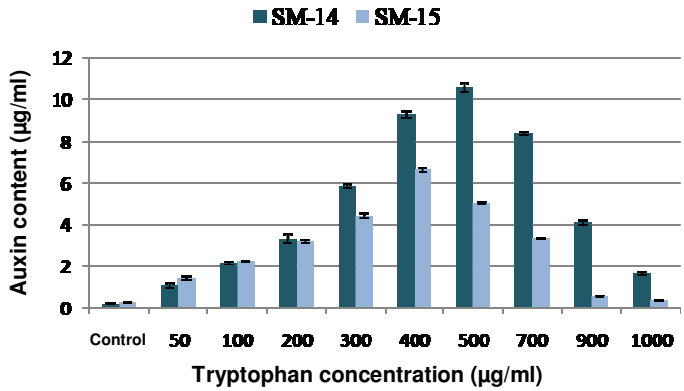

Figure 1. Colorimetric quantification of auxin in cyanobacterial strains in media supplemented with different concentrations of tryptophan.

was found to be 25°C and 7, respectively.

Strains identification

Recently, molecular-based tools are used in research and diagnostic laboratories for identification purposes. 16S rRNA gene sequencing is the most frequently used method for microbe identification, as it is the least variable gene sequence (Fredricks and Relman, 1996; Kolbert and Persing, 1999; Kiratisin, 2003). Therefore, in present study, 16S rRNA gene sequence analysis was carried out and compared with the NCBI sequence database (GenBank) through BLAST (www.ncbi.nlm.nih.gov/BLAST). On the basis of sequence identity, strains SM-14 and SM-15 were 99 and 100% similar, respectively, with *Phormidium*. Sequence length and GenBank accession numbers of the strains are presented in Table 2.

Plant growth promoting traits

Both cyanobacterial strains showed positive results for auxin production, nitrogen fixation, phosphate solubilization and hydrogen cyanide production (Table 3). Both strains showed growth in nitrogen free media. Phosphorus and nitrogen are very essential nutrients for plant growth and inoculation with phosphate solubilizing and nitrogen fixing cyanobacteria has been shown to improve plant growth by increasing the availability of phosphate and nitrogen content (Natesan and Shanmugasundaram, 1989; Svircev et al., 1997; Hameeda et al., 2008). Cyanobacterial strains may protect plants from phytopathogens due to hydrogen cyanide production (Ahmad et al., 2008). Phytohormone producing cyanobacteria are also involved in the promotion of plant growth. We further checked the auxin production of cyanobacteria with well established colorimetric method. Moreover, colorimetric analysis of cyanobacterial cultures showed variable amount of auxin production in the absence and presence of different concentration of L-Tryptophan. SM-14 showed high amount of auxin in the media supplemented with 500 µg/ml Tryptophan whereas in case of SM-15 strain high amount of auxin production was observed with 400 µg/ml Tryptophan (Figure 1). It was also noted that at very high concentrations of Tryptophan, auxin production decreases.

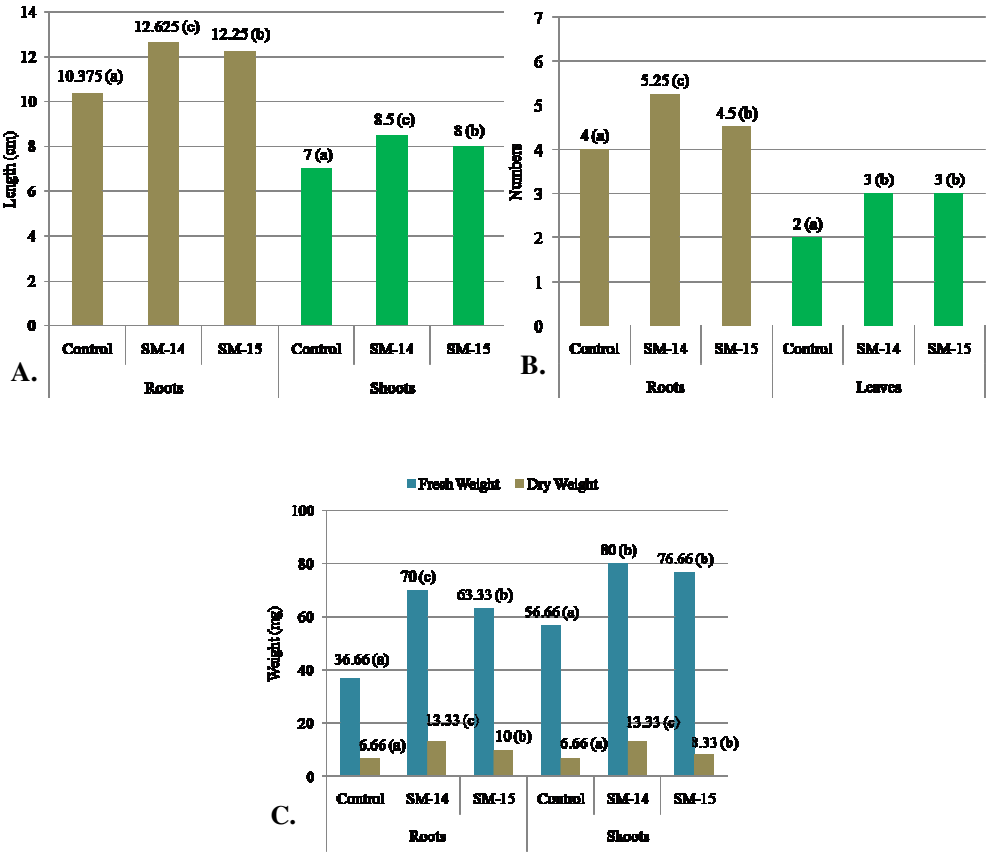

Figure 2. Effect of cyanobacterial inoculation on the length (A), number (B) and weight (C) parameters of *T. aestivum* var. Uqab 2000. Mean of twelve replicates, different letters within parenthesis indicate significant difference between treatments for each parameter using Duncan's multiple range test (P = 0.05).

Plant growth experiments

Effect of cyanobacterial strains on the growth of *T. aestivum* var. Uqab 2000 was studied by inoculating the seeds with cyanobacterial cultures. The results of inoculated and non-inoculated plants were recorded in order to make comparison. It was observed that inoculation with the cyanobacterial strains stimulated growth of *T. aestivum* var. Uqab 2000. Many researchers have reported that the co-cultivation of crops with cyanobacteria caused a considerable increment in growth and biochemical parameters, both in control and field conditions (Karthikeyan et al., 2008; Osman et al., 2010; Begum and Islam, 2011). In the current study, almost 14 and 21% increase in the shoot length and 18 and 21% increase in root length of the inoculated plants was observed with SM-15 and SM-14 inoculation, respectively, as compared to non-inoculated treatments. Our results agree with those obtained by the cyanobacterial inoculation of wheat (Mohiuddin et al., 2000; Karthikeyan et al., 2007), *Lupinus termis* (Haroun and Hussein, 2003), pea (Osman et al., 2010) and rice (Nilsson et al., 2002; Saadatnia and Riahi, 2009; Begum et al., 2011). The increase in root and shoot lengths of wheat seedlings could be due to the action of one or more of the growth promoting substances especially auxins (Sergeeva et al., 2002; Prasanna et al., 2010) and cytokinins (Ordog et al., 2004; Hussain et al., 2010) present in the cyanobacteria. The phytostimulatory potential of cyanobacteria was also attributed to the atmospheric nitrogen fixation, making it available to the associated plants (Karthikeyan et al., 2007). Increase in root length and surface area affects the overall development and growth of the plants by stimulating the water and nutrient uptake from soil. Both isolates improved plant growth by 50% increase in the number of leaves over the control treatment. Both the strains also increased the number of roots (12. 5 to 31.25% increase in the number of roots caused by SM-15 and SM-14 over control treatment respectively). Cyanobacterial strain caused increment in the fresh and dry weight of shoot and root over the respective control treatment (Figure 2). The increment in fresh weight of shoot ranged from 72 (SM-15) 90% (SM-14) and of root ranged from 50 (SM-

15) to 100% (SM-14). Almost 35 and 41% increase in the dry weight (mg) shoot and 25 and 100% increase in dry weight (mg) of root of the inoculated plants was observed with SM-15 and SM-14 inoculation, respectively, as compared to non-inoculated treatments. From the present study, we concluded that both cyanobacterial strains stimulated the growth of plant and they can be effectively used for biofertilization and plant growth improvement of different crops.

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