# Plant Growth Promoting Activities of Cyanobacteria Growing In Rhizosphere of Agriculturally Fertile Soil

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**Abstract:** Cyanobacteria are gram negative oxygenic photo synthesizer prokaryotes and are considered as an important group of microorganisms capable of fixing atmospheric nitrogen.

In the present investigation fifteen cyanobacteria were isolated from the rhizosphere of rice fields. Of these, the heterocystous isolates possess high levels of chl-a, nitrogenase, total nitrogen, IAA, P-solubilization and siderophore production. Hence, heterocystous cyanobacterial isolates have greater capacity to fix dinitrogen, to produce IAA, siderophores and P-solubilization, and might proved to be effective biofertilizers for agricultural crops.

Key Words: Cyanobacteria, rhizosphere, Nitrogenase, IAA, Siderophore, P-solubilization

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#### I. Introduction

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Cyanobacteria are gram negative oxygenic photo synthesizer prokaryotes commonly found in fresh water, marine water and soil. They are considered as an important group of microorganisms capable of fixing atmospheric nitrogen. They have a unique potential to contribute to productivity in a variety of agricultural and ecological situations. Many cyanobacteria fix nitrogen under aerobic conditions in specialized cells called heterocyst which comprise 5-10% of cells in a filament [1] (Gantar, 2000). Nonheterocystous cyanobacteria are also able to promote plant growth and can also be used as bio fertilizer.

Besides fixing atmospheric nitrogen, cyanobacteria play a major role in reducing soil erosion because of ability to secrete polysaccharides that bind soil [2] (Nayak and Prassana, 2007). They also control soil run off and increase soil organic matter content and in producing certain substances which enhance the growth of plants [3] (Ordog, 1999). Due to this important characteristic of nitrogen fixation, the utility of cyanobacteria in agriculture to enhance production is beyond doubt.

Cyanobacteria evolved very early in the history of life, and share some of the characteristics of gliding bacteria on one hand and those of higher plants on the other. Cyanobacteria also have a unique potential to contribute to productivity in a variety of agricultural and ecological situations. Cyanobacteria have been reported from a wide range of soils, thriving both on and below the surface.

A diverse group of microorganisms are found to be associated with the rhizosphere of plants. These microorganisms are known to produce metabolites, which can have beneficial, harmful or neutral effects on the plants [4, 5, 6] (Barriuso *et al.*, 2008; Berendsen *et al.*, 2012; Mendes *et al.*, 2013). In the rhizosphere the plant growth promoting micro-organisms (PGP) release signaling molecules called elicitors, which can bind to receptor sites on plasma membrane and trigger signal cascades that can cause activation of genes, leading to the production of proteins and enzymes or synthesis of secondary metabolites [7, 8, 9, 10, 11] (Nurnberger *et al.*, 1994; Zhao and Sakai, 2003a, b; Zhao *et al.*, 2004, 2005).

Cyanobacteria are prominent inhabitants of many agricultural soils where they potentially contribute towards biological nitrogen fixation, help in phosphate solubilization and mineral release to improve soil fertility and crop productiovity. However, besides naturally fertilizing and balancing mineral nutrition in the soil, many cyanobacteria are known to release various kinds of biologically active substance like carbohydrates, proteins, vitamins, amino acids and phytohormones, which function as signaling molecules to promote plant growth [12, 13] (Zaccaro *et al.*, 1991; Mandal *et al.*, 1998).

The role of cyanobacteria in the enhancement of rice yield has been well documented in literatures [14, 15] (Watanabe, 1951; Rodgers *et al.*, 1979). The cyanobacterial species viz. *Nostoc, Anabaena, Calothrix, Hapalosiphon, Oscillatoria, Lyngbya, Phormidium*, isolated from the rhizosphere of diverse rice and wheat varieties had been tested for their plant-growth promoting properties on crops grown in pots [16, 17, 18] (Obana *et al.*, 2007; Prasanna *et al.*, 2009a; Prasanna et al., 2013b).

The increase in growth parameters of crops like germination rate, shoot length, root length and biomass had been shown to be positively correlated with an increase in various phytochemical constituent of the plant. Rice plant inoculated with Cyanobacterial strains, viz. Anabaena oryzae, Anabaena dolium, Phormidium fragile, Calothrix geitons, Hapalosiphon intricatus, Aulosira fertilissima, Tolypothrix tenuis, Oscillatoria acuta and Plectonema boryanum showed accumulation of phenolic acids (gallic, gentisic, caffeic, chlorogenic and ferulic acids), flavonoids (rutin and quercitin), phytohormones (indole acetic acid and indole butyric acid), protein and chlorophyll in the leaves [19] (Singh et al., 2011). The bioactive compounds of cyanobacteria were shown to increase the levels of phytohormones, which is responsible to trigger root and shoot growth of plant. Phytohormones are also known to regulate several enzymatic activities and the metabolic changes occurring during plant growth [20, 21] (Tsai et al., 1997; Tvorogova et al., 2013), and therefore, the increase in the activity of defence enzymes, peroxidase and phenylalanine ammonia lyase could also be attributed to the levels of phytohormenes. Phenolics, especially flavonoids and phytohormones are shown to help in plant-microbe interactions [22, 23] (Hirsch et al., 2010; Cooper, 2004), enhance root colonization by microbes [24] (Narula et al., 2006), promote allelochemical influence on population of other organisms [25, 26] (Hooper et al., 2011; Cushine and Lamb, 2011) and also act as signal molecules [27, 28] (Hartwig and Phillips, 2013; Stamm and Kumar, 2010).

The role of cyanobacteria as biofertilizer has largely been reviewed by [29, 2, 3, 30, 31, 32] Dola Bhowmik et al., (2010), Nayak and Prassana (2007), Ordog (1999), Haroun and Hussein (2003), Lakshmi and Annamalai (2008), Gallab and Salem (2001). An additional benefit of cyanobacterial consortium with crops is their capacity to secrete bioreactive substances such as auxins, gibberrelins, cytokinins, vitamins, polypeptides, aminoacids, etc. which promote plant growth and development. The plant growth promotory effect on cow pea (*Vigna unguiculata*) using coir pith aqueous extract formulation of cyanobacterium *Phormidium* has been investigated by [33] Pitchai Palaniappan et al., (2010). Plant growth promoting substances produced by cyanobacterial consortium with crops have largely been reviewed by [34, 35, 36] Karthikeyan et al., (2007), Prasanna et al., (2008), Fatima and Venkataraman (1999) etc. Effect of exopolysaccharides (EPS) produced by a consortium of cyanobacteria of three crops, wheat, rice and maize have been studied by [37] Manu Arora et al., (2010).

The present study has been undertaken to evaluate the plant growth promoting capacity of some heterocystous and non-heterocystous cyanobacteria *in vitro* in terms of nitrogenase activity and total nitrogen, chlorophyll-a and phytohormones (Indole acetic acid), P- solubilization and siderophore production.

## **II.** Materials and Methods

The present work was carried out in the Microbiology research laboratory, Dept. of Biotechnology, College of Commerce, Patna from March to June 2019. The Cyanobacterial flora were isolated from rhizosphere of rice grown in shallow water and identified by relevant monographs [38, 39, 40, 41] (Desikachary, 1959; Tilden, 1910; 1937; Hegewald, 1976). The cyanobacterial samples were maintained in pure culture in BG11 medium in a growth chamber under12/12hL/D cycle at 25±2°C and 1500 lux light intensity using fluorescent lamps.

Fifteen cyanobacterial flora were isolated from rhizosphere of rice plants viz., Oscillatoria nigra, O. princeps,O. curviceps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum, Calothrix clavata, Aulosora prolifica, Stigonema dendroideum, Nostoc muscorum, Nostoc calcicola, Anabaena oryzae, Scytonema varium, Gloeocapsa calcarea and Tolypothrix tenuis. The nitrogenase activity, total nitrogen, chlorophyll-a, IAA, P-solubilization and siderophore production by cyanobacterial isolates were assayed *in vitro*. The chlorophyll-a content was measured by the method suggested by [42] Mackinney (1941).

The ability of cyanobacteria to fix dinitrogen was measured by acetylene reduction assay i. e. the ability of cyanobacterial nitrogenase complex to reduce acetylene ( $C_2H_2$ ) to ethylene ( $C_2H_4$ ). Approximately 1g of air-dried cyanobacterial isolates, obtained from free living cultures was dispensed into 25ml glass vials. Sterilized distilled water was added to give a total volume of 7.5ml. The vials were sealed with screw caps fitted with silicon rubber septa. Acetylene generated from calcium carbide was injected into the vials, giving a gas atmosphere in the vials of air plus 10% acetylene. All samples were set up in duplicate sets of three each for light and dark incubations; the latter for assessing dark nitrogen fixation. After incubation for specific duration in light or dark according to the requirement of the experiment, 100µl of gas phase was withdrawn using a gastight Hamilton syringe and analyzed for ethylene on AIMIL- Nucon 5765 model gas chromatograph with FID detector fitted with Porapak- T SS column (80 100 mesh; carrier gas nitrogen, 30ml/min; column temperature, 100°C; injector temperature, 100°C; detector temperature, 120°C). Acetylene reduction activity was expressed as n mol ethylene /g air dried material/h [43] (Umbreit *et al.*, 1972).

The total nitrogen content of free living cyanobacterial isolates was estimated according to modified micro-Kjeldahl method (Nesslerisation) of Umbreit *et al.*, (1972) [43]. Total nitrogen in cyanobacterial culture filtrate was carried out in two phases:

**Digestion phase**: A suitable amount (about 50ml) of culture filtrate was taken in Kjeldahl flask and to this added 50ml of digestion reagent. Digestion reagent was prepared by dissolving 134gK2SO4 in 650ml of distilled water and 200ml of concentrated  $H_2SO_4$ . 2g HgO was dissolved in 6NH<sub>2</sub>SO<sub>4</sub>. This solution was then added to test solution. The solution was diluted to 1 liter and stored at temperature above 14°C to prevent crystallization.

**Distillation phase**: Kjeldahl flask was then placed in its proper position in distillation apparatus and turned on hear. 0.5ml of phenolphthalein indicator followed by sodium hydroxide and sodium thiosulphate was added till pH reach just above 8.3. After distillation 200ml of distillate was collected in 50ml boric acid. On completion of distillation the flask was removed and put off heat to avoid back suction. The concentration of ammonia was measured by nesslerization. The distillate was then titrated with 0.02N H2SO4 till the indicator turned a pale lavender color. The total nitrogen (organic nitrogen) was calculated by following formula:

# Total Nitrogen = (A-B) x280/ml of sample

Where A = ml of 0.02N H<sub>2</sub>SO<sub>4</sub> used with sample and B = ml of 0.02N H<sub>2</sub>SO<sub>4</sub> used with blank.

To measure the amount of Indole acetic acid (IAA) produced by cyanobacterial isolates, 1.5 ml of culture broth (prepared in BG-11 medium) was centrifuged at 12000 rpm for 5 minutes. One milliliter of the supernatant was added to 2 ml FeCl<sub>3</sub>-HClO<sub>4</sub> reagent. After 25 minutes the absorbance was recorded at 530 nm in UV-spectrophotometer. The amount of IAA produced per milliliter was estimated using a standard calibration curve.

Phosphate-solubilization activity of cyanobacterial isolates was conducted qualitatively by plating the cyanobacteria in agar BG-11 medium supplemented with precipitated tricalcium phosphate. The presence of clear zone around the cyanobacterial colonies after overnight incubation indicated the phosphate solubilization activity of cyanobacteria.

Siderophore production by cyanobacterial isolates was assayed qualitatively using chrome azurol S (CAS) agar. The CAS agar was a mixture of four solutions that were prepared separately before mixing. Solution-1 (Fe-CAS indicator solution) consisted of 10 ml of 1 mM FeCl3.6H2O (in 10 mM HCl), 50 ml of an aqueous solution of CAS (1.21 mg/ml), and 40 ml of aqueous solution of hexadecyl-trimethylammonium bromide (HDTMA) (1.82 mk/ml). Solution-2 (buffer solution) was prepared by dissolving 30.24 g of PIPES (Peprazine-N, N'-bis[2-ethansulphonic acid]) in 750 ml of salt solution. The distilled water was added to bring the volume to 800 ml after adjusting the pH to 6.8 with 50% KOH. The solution was autoclaved after adding 15 g agar. Solutuon-3 contained 2 g glucose, 2 g mannitol, and trace elements in 70 ml distilled water. Solution-4 was 30 ml filtered-sterilized 10% (w/v) casamino acid. At 50<sup>o</sup>C after autoclaving, solution-3 and 4 were added to the buffer solution. Indicator solution (solution-1) was added last with sufficient stirring to mix the ingredients. This mixture (Fe-CAS dye complex) yielded blue to dark green colour. The cyanobacterial culture of each isolate was spreaded on the CAS agar plates with two replications. Orange halos around the colonies after overnight incubation indicated siderophore production by cyanobacteria. The results obtained have been presented in Table-1 and 2; Figure-1-4.

Cyanobacterial isolates	Chlorophyll-a (µg/ml)	Nitrogenase (n moles	Total nitrogen (µg/mg dry
		C <sub>2</sub> H <sub>4</sub> produced/hr/mg dry wt)	wt)
Oscillatoria curviceps	0.145	1.3	0.415
O. nigra	0.277	0.7	0.417
O. princeps	0.154	0.8	0.418
Schizothrix vaginata	0.143	1.2	0.415
Lyngbya gracilis	0.146	1.3	0.416
Phormidium dimorphum	0.135	1.5	0.423
Calothrix clavata	0.145	1.4	0.419
Aulosira prolifica	0.431	2.5	0.840
Stigonema dendroidum	0.445	2.7	0.847
Nostoc muscurum	0.456	3.2	0.853
N. calcicola	0.465	3.5	0.851
Anabaena oryzae	0.470	5.5	0.870
Scytonema varium	0.462	5.4	0.866

Table-1: Production of Chlorophyll-a, Nitrogenase and Total nitrogen by cyanobacterial isolates

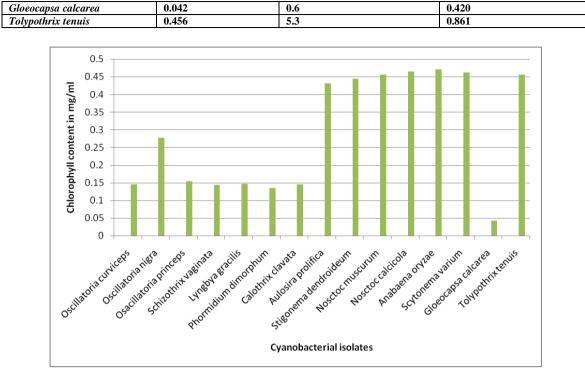


Figure-1: Chlorophyll-a content in 15 cyanobacterial isolates in mg/ml

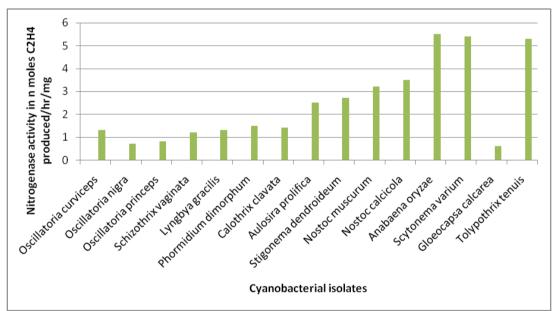


Figure-2: Nitrogenase activity of 15 cyanobacterial isolates in n moles C<sub>2</sub>H<sub>4</sub> produced/hr/mg of dry wt.

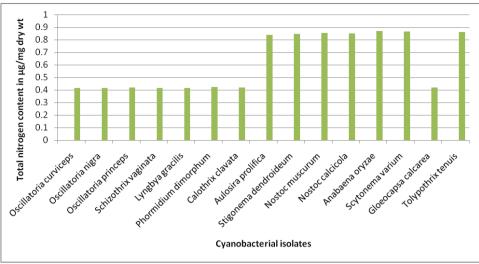


Figure-3: Total nitrogen content of 15 cyanobacterial isolates in  $\mu$ g/mg of dry wt.

Table-2: IAA production, P- solubilization and Sideroph	hore production by cyanobacterial isolates
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Cyanobacterial isolates	IAA (µmole/ml)	P-solubilization)	Siderophore production
Oscillatoria curviceps	nd	-	-
O. nigra	nd	-	-
O. princeps	nd	-	-
Schizothrix vaginata	nd	-	-
Lyngbya gracilis	nd	-	-
Phormidium dimorphum	nd	-	-
Calothrix clavata	nd	-	-
Aulosira prolifica	34.35	+	+
Stigonema dendroidum	35.45	+	+
Nostoc muscurum	36.75	+	+
N. calcicola	35.85	+	+
Anabaena oryzae	38.35	+	+
Scytonema varium	34.65	+	+
Gloeocapsa calcarea	nd	-	-
Tolypothrix tenuis	33.25	+	+

Nd = not detected; + = present; - = absent

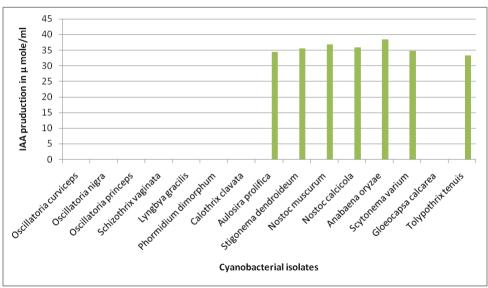


Figure-4: Production of IAA in µmole/ml by 15 cyanobacterial isolates

## **III. Results**

From the result (Table-1; Fig-1) it is evident that the chl-a content of heterocystous cyanobacteria viz., *Anabaena oryzae, Scytonema varium, Tolypothrix tenuis, Nostoc calcicola, N. muscurum, Stigonema dendroideum* and *Aulosira prolifica* was maximum in free living condition, in the range of 0.470 to 0.431µg/ml and minimum in non heterocystous forms (0.042 to 0.277µg/ml).

The Nitrogenase content in free living cyanobacteria was maximum in heterocystous cyanobacteria viz., Aulosira prolifica, Stigonema dendroideum, Nostoc muscurum, N. calcicola, Anabaena oryzae, Scytonema varium and Tolypothrix tenuis in the range of 0.580 to 0.590 n moles C2H4 produced/h/mg dry wt, and minimum in non-heterocystous forms (0.310 to 0.353 n moles  $C_2H_4$  produced/h/mg dry wt), viz., Oscillatoria curviceps, O. nigra, O. princeps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum and Gloeocapsa calcarea. The results clearly indicated that non heterocystous cyanobacteria also had the capacity to fix atmospheric nitrogen but to a lesser extent. Among 15 cyanobacterial isolates the total nitrogenase content was found to be minimum in Gloeocapsa calcarea (0.6µg/ml dry wt.), O.nigra (0.7µg/ml) and O. princeps (0.8µg/ml) and maximum in heterocystous forms viz., Anabaena oryzae (5.5µg/ml), Scytonema varium (5.4µg/mg), Tolypothrix tenuis (5.3µg/mg), Aulosira prolific (2.5µg/mg), Stigonema dendroideum (2.7µg/mg), Nostoc muscurum (3.2µg/mg) and N. calcicola (3.5µg/mg) in free living state. Oscillateria curviceps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum, Colothrix clavata showed mild nitrogenase concentration in their free states (Table-1; Fig-2).

The total nitrogen content was higher in heterocystous cyanobacteria viz. *Aulosira prolifica, Stigonema dendroideum, Nostoc muscurum, N. calcicola, Anabaena oryzae, Scytonema varium* and *Tolypothrix tenuis* in the range of 0.840 µg/mg to 0.870 µg/mg of dry wt in comparison to non-heterocystous isolates viz. *Oscillatoria curviceps, O. nigra, O. princeps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum* and *Gloeocapsa calcarea* in which the total nitrogen content ranged between 0.415 and 0.420 µg/mg dry wt. (Table-1; Fig-3).

In non-heterocystous cyanobacterial isolates viz. *Oscillatoria curviceps, O. nigra, O. princeps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum* and *Gloeocapsa calcarea* the production of IAA was not detected. Similarly, Phosphate solubilization and siderophore production were found to be absent by these non-heterocystous isolates. Heterocystous cyanobacterial isolates viz. *Aulosira prolifica, Stigonema dendroideum, Nostoc muscurum, N. calcicola, Anabaena oryzae, Scytonema varium* and *Tolypothrix tenuis* showed the production of IAA, siderophore and compounds responsible for phosphate solubilization (Table-2; Fig-4). Among heterocystous cyanobacterial isoletes, the IAA level was maximum in *Anabaena oryzae* (38.35 µmole/ml), followed by *Nostoc muscurum* (36.75 µmole/ml), *Nostoc calcicola* (35.85 µmole/ml), *Stigonema dendroideum* (35.45 µmole/ml), *Scytonema varium* (34.65 µmole/ml), *Aulosira prolifica* (34.35 µmole/ml) and *Tolypothrix tenuis* (33.25 µmole/ml) (Table-2; Fig-4).

### **IV. Discussion**

The use of cyanobacteria as a biofertilizers for rice crops was reported by [44, 45, 46, 47, 48, 49] De (1939), Kannaiyan (1979), Roger and Kulassariya (1980), Roger (1996), Anand Mohan *et al.*, (2015), Manoj Kumar *et al.*, (2013) etc.

N fixing ability of these heterocystous cyanobacteria can increase the content of easy available N or the ammonium content of algae extracts. According to the influential role of N fixation in the N content of cyanobacterial extract. The greater nitrogen fixing capacity influences greater improvement of plant growth [50, 51, 52] (Gantar et al., 1995; Irisarri et al., 2001; Nilsson et al., 2002). The present investigation revealed that the presence of some growth-promoting substances viz. nitrogenase, IAA, siderophores and P-solubilization may be responsible for the beneficial effect on plant growth parameters. The results showed the presence of IAA, P-solubilization and siderophore in heterocystous cyanobacteria (Table-2) which might be responsible for the enhanced growth of plants [53, 54, 55, 56] (Lee and Hackett 1976; Simpson 1986; Mahmood Khavar and Özcan 2002; Mobli and Baninasab 2009). Li et al., (2009) [57] also showed that phytohormones can promote root elongation and ion uptake in rice seedlings. A positive effect of heterocystous cyanobacteria on plant growth and nutrient uptake was reported by [58] Obana et al., (2007). They showed that *Nostoc* application increased the organic C and N content of the surface soil and enhanced plant growth and plant ion uptake. They believed that the microelements necessary for plant growth can be supplied by these microorganisms. In addition, polysaccharides secreted by cyanobacteria contribute to the structural stability of the soil, to increased soil C and N levels, and to the promotion of plant growth [59] (Foth 1990).

Hashtroudi *et al.*, (2013) also reported the production of indole-3-acetic acid (IAA) and two of its main homologues indole-3-propionic acid and indole-3-butyric acid from the heterocystous cyanobacterial strains of *Anabaena vaginicola* and *Nostoc, calcicola*. From the work of Hashtroudi *et al.*, (2013) [60] it seems that similar to higher plants, IBA may be converted to IAA to maintain the auxin level, and it has a possible role as an auxin storage form in cyanobacteria [61] (Koshitaa *et al.*, 1999).

Phytohormone production had been reported mostly from cyanobacteria isolated from crop fields. However, a mangrove root-associated cyanobacterium, *Phormidium* sp. has been demonstrated to produce IAA [62] (Boopathi *et al.*, 2013). The extracellular extract of this cyanobacterial culture filtrate was tested on tobacco seeds and was found to enhance seed germination and callus differentiation. Extract-treated callus showed multiple root formation, a characteristic feature of auxins. There are many reports showing that IAA producers increase root mass and length [63, 64, 65] (Casimiro *et al.*, 2001; Péret *et al.*, 2009; Overvoorde *et al.*, 2010), which helps the plants with enhanced access to soil nutrients. In addition, IAA loosens the plant cell wall, increasing the root exudation that provides additional nutrients to the producer [66] (Edelmann and Kutschera, 2009).

Growth-promoting phytohormones viz. cytokinin and indole-3-acetic acid (IAA) have been detected in cyanobacterial strains of *Anabaena*, *Oscillatoria*, *Phormidium*, *Chroococcidiopsis*, *Synechocystis* [67, 68] (Hussain *et al.*, 2010; Hussain and Hasnain, 2012).

#### V. Conclusions

Cyanobacteria are ubiquitous in agricultural soils. They are the primary microbial photosynthetic agents of the soil. Due to the important characteristic of nitrogen fixation, the utility of cyanobacteria in agriculture to enhance production is beyond doubt. Considerable progress has been made in the development of cyanobacterial based biofertilizer as a means of aiding fertility to the soils. Their presence in soils is of great importance for the functioning of many processes. It has been observed that cyanobacterial inoculation in crop fields has shown to bring yield improvement even in the presence of high doses of nitrogenous fertilizers. Hence, it is important to obtain a much more detailed understanding of cyanobacterial population dynamics in agriculture systems and understand the abundance of these micro-organisms in agricultural fields.

Besides enhancing nitrogen fertility, cyanobacteria use elicitor signalling as a tool for plant growth promotion. The biosynthesis of phytohormones, polysaccharides, vitamins, amino acids and peptides are considered to be crucial for plant growth and development. They release these active compounds in the rhizosphere from where plant roots may absorb them.

Cyanobacteria form symbiotic association with members of all major plant groups. However, they are of little economical as none are crop plants. There are no natural symbioses between cyanobacteria and crop plants. Because of its tremendous beneficial effects on crop plants, a great deal of interest is currently diverted in making simulated associations of cyanobacteria with domesticated plants to improve plant growth and yield, as well as minimize need for chemical fertilizers. Certain progress had been made in developing artificial associations between diazotrophic cyanobacteria and important agricultural plants so that a continuous interaction throughout the period of crop growth is provided. From the present investigation it can be concluded that the heterocystous cyanobacterial isolates have greater capacity to produce nitrogenase, IAA, siderophores and P-solubilization, and might proved to be effective biofertilizers for agricultural crops.

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