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Original Research Article

Characterization of Cyanobacteria for IAA and Siderophore Production and their Effect on Rice Seed Germination

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Keywords

Cyanobacteria, Plant growth promotion, IAA, Siderophore

A B S T R A C T

The practical importance of cyanobacteria as a source of nitrogen fertilizer in rice is well documented. Five cyanobacterial cultures *viz. Anabaena variabilis* (CCC441), *Nostoc muscorum* (CCC442), *Tolypothrix tenuis* (CCC443), *Aulosira fertilissima* (CCC444) and *Westelliopsis prolifica* (CCC128) maintained in Culture Collection of Cyanobacteria (CCC) at CCUBGA, Indian Agricultural Research Institute, New Delhi, India were taken. Cell free supernatant of 14 days old cultures showed germination percent in the range of 93 -96 % whereas the cell free supernatant of 21 day old cultures gave 96-99 % germination after 72 hour as compared to 92-95% germination in control. There was variable response in terms of shoot growth and root growth of seedlings of rice in presence of cell free supernatant. Shoot length (cm) ranged from 3.54-5.42 cm with *Nostoc muscorum* (CCC442) showing maximum enhancement in shoot length (5.42cm) followed by *Anabaena variabilis* (CCC441) 5.25cm. There was more stimulation of root growth of rice seedlings by cell supernatant of *Anabaena variabilis* (CCC441) which showed maximum 7.15 cm followed by *Nostoc muscorum* (CCC442) and *Westelliopsis prolifica* (CCC128). The strains were positive for IAA and siderophore production reaffirming the importance of these organisms in agriculture. *Nostoc muscorum* (CCC442) followed by *Anabaena variabilis* (CCC441) was most consistent and may have the potential for development as a plant growth promoting inoculant.

Introduction

Cyanobacteria, also known as blue green algae, are the prokaryotic group of organism which constitutes a major part of phytoplankton and a primary colonizer in different habitats. The practical importance of cyanobacteria as a source of nitrogen fertilizer in rice is well recognized (Singh and Singh, 1989) and plays a significant role in sustaining and improving rice field productivity (Rodger *et al.*, 1979; Pabbi, 2008). Earlier it was believed that higher yield was due to nitrogen fixation only. But a number of studies showed that improved yield is not only due to nitrogen fixation but also due to production of growth promoting substances (Venkataraman and Neelakantan, 1967; Marsalek *et al.*, 1992), phosphate solubilising ability (Bisoyi and Singh, 1988), addition of organic matter (Singh and Bisoyi, 1989; Das *et al.*, 1991), pesticide tolerance (Ahmed and Venkataraman, 1973; Kaushik and Venkataraman, 1983, Pabbi

and Vaishya, 1992) and improvement in physical properties of soil (Bertocchi *et al.*, 1990; Roger and Burns, 1994). It was also found that cyanobacteria liberate several extra-cellular products like growth promoters, amino acids, vitamins, useful enzymes, sugars etc. (Singh and Trehan, 1973; Malliga *et al.*, 2002; Prasanna *et al.*, 2008a) which have direct or indirect impact on plant growth and yield. Cyanobacteria have also been reported to produce bioactive compounds including plant growth regulators. Manickavelu *et al.*, (2006) reported growth regulating effects of cyanobacterial extracellular product on organogenesis induction in rice callus. In tissue culture, extracts of cyanobacteria *Plectonema* sp. has been reported to stimulate somatic embryogenesis and somatic embryo development in Sandalwood (*Santalum album*) (Bapat *et al.*, 1996). Cyanobacterial biomass extract have been observed to promote seed germination (Pedurand and Reynaud, 1987), somatic embryogenesis (Wake, 1992) and root induction in rice callus (Manickavelu *et al.*, 2006). Auxins represent a group of plant hormones that are implicated in the regulation of diverse biological processes including cell division, elongation, differentiation and root elongation. Among the auxins, IAA (indole-3-acetic acid) is recognized as a key factor, which is directly beneficial for plants (Costacurta and Vanderleyden, 1995). The ability of cyanobacteria to produce phytohormone, IAA, has been demonstrated (Sergeeva *et al.*, 2002; Prasanna *et al.*, 2010). Although there are not many studies involving IAA synthesis by cyanobacteria but their ability to stimulate and facilitate plant growth reaffirms their potential to produce many naturally occurring phytohormones. The IAA biosynthesis has been found in representatives of free living and symbiotic cyanobacteria belonging to the genera *Nostoc, Chlorogleopsis, Calothrix, Plectonema, Gleothece, Anabaena, Cylindrospermum* and *Anabaenopsis*. Misra and Kaushik (1989) have reported the production of growth promoting substances by *Nostoc* and *Hapalosiphon* and *Hapalosiphon* extract was found to contain IAA with the possibility of presence of indole-3-propionic acid or 3-methyle indole. Both tryptophan independent pathway which is more common in plants and tryptophan dependent pathway have been reported in cyanobacteria (Prasanna *et al.*, 2010). Cyanobacteria with their complex life cycle and their unique ability of N_2 fixation appear to share some mechanism for accumulation of IAA with some other plant interacting bacteria as well as plants. Similarly, the ability of cyanobacteria to produce siderophores has been known for some time.

Estep *et al.*, (1975), Murphy *et al.*, (1976), Simpson & Neilands (1976) and Armstrong and Van Baalen (1979) were among the first to demonstrate that cyanobacteria were capable of siderophore production during iron limitation. One of the best-studied systems is that of schizokinen, a citrate derivative siderophore from the freshwater cyanobacterium *Anabaena* PCC 7120 (Gademann and Portmann, 2008). It has been reported that hydroxamate- type iron chelators produced by cyanobacteria during periods of iron limitation (Boyer *et al.*, 1987; Trick and Kerry, 1988). Brown and Trick (1992) reported that *Oscillatoria tenuis* is the only cyanobacterium to date that produces catechol-type siderophores. Gilliam *et al.*, (1981) used the procedures of Czaky to determine the chemical nature of the iron chelators isolated from the cyanobacteria and Arnow (1937) to test for respective hydroxamate- and catechol-type siderophores. But this group of prokaryotes has not been investigated fully in terms of their plant growth promoting potential

especially in relation to production of phytohormones and siderophores. The present investigation was aimed towards characterizing the growth promoting potential of selected cyanobacterial strains for production of IAA and siderophore with a view of developing inoculants having diverse applications.

Materials and Methods

Organisms and Growth conditions

Five cyanobacterial cultures *viz*. *Anabaena variabilis* (CCC441), *Nostoc muscorum* (CCC442), *Tolypothrix tenuis* (CCC443), *Aulosira fertilissima* (CCC444) and *Westelliopsis prolifica* (CCC128) maintained in Culture Collection of Cyanobacteria (CCC) at CCUBGA, Indian Agricultural Research Institute, New Delhi were taken for the study. The cultures were grown and maintained in chemically defined BG-11 (-N) medium (Stanier *et al.*, 1971) in a culture room at 28 ± 2^{o}C under a light intensity of 52-55 µmol photon m^{-2} s $^{-1}$ and L: D cycles of 16:8 hours.

Seed germination and growth assay

5 ml of (14th day and 21st day old) cyanobacterial cell free supernatant were bio-assayed for their ability to promote/inhibit seed germination and seedling growth. The seeds of rice (Pusa Basmati 1509) were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi.After treatment with 0.1 % $HgCl_2$ for 3 min, the seeds were placed on seedling agar (1% water agar) and kept at 28°C in dark for germination. After 4 days, percent of germination, length (cm) of shoot and root were measured. Selected cyanobacterial strains based on their ability to promote both germination, shoot length and root length of rice seedlings were further checked for their ability of IAA and siderophore production to correlate their growth enhancing ability.

Percentage germination (%)

The percentage germination was calculated by dividing number of seeds germinated to the total number of seeds sown.

Estimation of IAA production (Gordon and Weber, 1951; Brick *et al.*, 1991)

On the basis of performance of cell free filtrates on seed germination/growth assay, the cyanobacterial strains were subjected to the following treatments in the presence of L-tryptophan at a concentration of 100, 200, 300, 400, 500 µg/ml in the medium.The experiment was done in triplicate with one set of control (without tryptophan). Samples were taken periodically at the intervals of 24, 48, 72 and 96 hr and analyzed for IAA production. The amount of IAA was quantified spectrophotometrically by measuring the intensity of pink color at 530 nm using standard calibration curve of IAA.

Estimation of Siderophore production (Schwyn and Neilands, 1987)

Each tested cyanobacterium was grown under both Fe-depleted (6 mg/l iron citrate, standard iron concentration in BG-11) and 1/10 Fe (0.6mg/l iron citrate) conditions. Liquid CAS assays as described by Schwyn and Neilands (1987) were conducted to detect the presence of siderophore in supernatants from the cyanobacterial cultures. The samples were allowed to equilibrate for 20 min at room temperature, and absorbance at 630 nm was measured. Reference estimation was done using uninoculated medium. The siderophore was quantified as % siderophore units increase (Payne, 1994).

Results and Discussion

Effect on seed germination and seedling growth

Evaluation of the cell free filtrates of five cyanobacterial cultures, in terms of their effect on germination of rice seeds was undertaken along with controls and14 day and 21 day old cell free supernatant of cyanobacterial cultures applied separately. All the cultures tested showed a positive effect on seed germination of rice. The germination percentage increased with increase in incubation time irrespective of culture or age of culture. The germination frequency was always more in presence of cell free supernatant as compared to control, i.e., BG 11 medium and sterile water. Percent of germination is ranging from 93 to 99%. *Nostoc muscorum* (CCC442) recorded maximum i.e.,99% followed by *Anabaena variabilis* (CCC441) and *Tolypothrix tenuis* (CCC443) which showed 98% whereas, *Aulosira fertilissima* (CCC444) and *Westelliopsis prolifica* (CCC128) showed 97% and 96% respectively as compared to 92 and 95% in the controls treatments(BG 11 medium and sterile water respectively) after 72 hour. Cell free supernatant of 14 day and 21 day old cultures did not vary much as far as percent germination was concerned. Cell free supernatant of 14 days old cultures resulted in germination percent in the range of 93 -96 % whereas the cell free supernatant of 21 day old cultures gave 96-99 % germination after 72 hour (Table 1). In terms of length of shoot and root recorded after 4 days, seeds soaked with cell free filtrates showed significantly higher values when 21 day old cell free supernatant was used than control. The length of root ranging from 5.25 to 7.15 cm. There was more stimulation of root growth of rice seedlings by cell supernatant of *Anabaena variabilis* (CCC441) which showed maximum 7.15 cm followed by *Nostoc muscorum* (CCC442) and *Westelliopsis prolifica* (CCC128) as compared to *Tolypothrix tenuis* (CCC443) and *Aulosira fertilissima* (CCC444). Contrary to the effect on root growth, the cell free supernatant of most cyanobacterial cultures had a positive effect resulting in enhancement of shoot growth of rice seedlings. Rice seedlings treated with the cell free supernatant showed shoot length (cm) ranging from 3.54-5.42 cm. *Nostoc muscorum* (CCC442) showed maximum enhancement in shoot length i.e., 5.42 cm followed by *Anabaena variabilis* (CCC441) 5.25cm, *Westelliopsis prolifica* (CCC128) 5.11cm and *Aulosira fertilissima* (CCC444) 4.10 cm after 4 days of inoculation. *Tolypothrix tenuis* (CCC443) did not show much enhancement in shoot length i.e., 3.96 as compared to control (3.65 and 3.55 cm by BG- 11 medium and sterile water respectively) (Table 2).

IAA production

The cyanobacterial cultures were evaluated for tryptophan dependent IAA production using varying concentration of L-tryptophan (100, 150, 300, 400 and 500µg/ml). All the cyanobacterial strains continuously accumulated IAA during the period of observation of 96 hr. Production of IAA was concentration dependent and increased with increasing concentration of L-tryptophan in the medium in case of *Nostoc muscorum* (CCC442), *Anabaena variabilis* (CCC441) and *Westelliopsis prolifica* (CCC128) whereas *Tolypothrix tenuis* (CCC443) and *Aulosira fertilissima* (CCC444) showed decreased production of IAA at highest concentration of tryptophan i.e., 500 µg/ml. Statistical analysis of the data confirmed that among all cultures, *Nostoc muscorum* (CCC442) produced highest amount of IAA closely followed by *Anabaena variabilis* (CCC441) (Figure 1 and 2).

Table.1 Effect of cell free supernatant of selected cyanobacterial strains on rice seed germination

Sl. No.	Culture	Seed germination (%) with					
		14th day supernatant			21st day supernatant		
		24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
1	*Anabaena variabilis* (CCC441)	80	90	95	80	85	98
2	*Nostoc muscorum* (CCC442)	75	85	96	80	90	99
3	*Tolypothrix tenuis* (CCC443)	70	90	93	75	85	98
4	*Aulosira fertilissima* (CCC444)	65	75	95	70	80	97
5	*Westelliopsis prolifica* (CCC128)	70	85	95	75	85	96
6	Control(BG-11)	60	80	92	60	80	92
7	Sterile water	65	85	95	65	85	95

[Results are the mean of 30 seeds (10 seeds per petriplates in triplicate) expressed in percentage]

Table.2 Effect of cell free supernatant of cyanobacterial strains on shoot and root length of rice seedlings after 4 days of inoculation

	Culture	Shoot length(cm)		Root length(cm)	
		14th day supernatant	21st day supernatant	14th day supernatant	21st day supernatant
1	*Anabaena variabilis* (CCC441)	4.19	5.25	5.8	7.15
2	*Nostoc muscorum* (CCC442)	3.86	5.42	5.58	6.95
3	*Tolypothrix tenuis* (CCC443)	3.6	3.96	5.54	5.98
4	*Aulosira fertilissima* (CCC444)	3.75	4.1	5.25	5.9
5	*Westelliopsis prolifica* (CCC128)	3.54	5.11	5.35	6.62
6	Control(BG-11)	3.6	3.65	5.2	5.78
7	Sterile water	3.5	3.55	4.45	4.45

Fig.1 IAA production by *Anabaena variabilis* (CCC441) in presence of different levels of tryptophan (µg/ml)

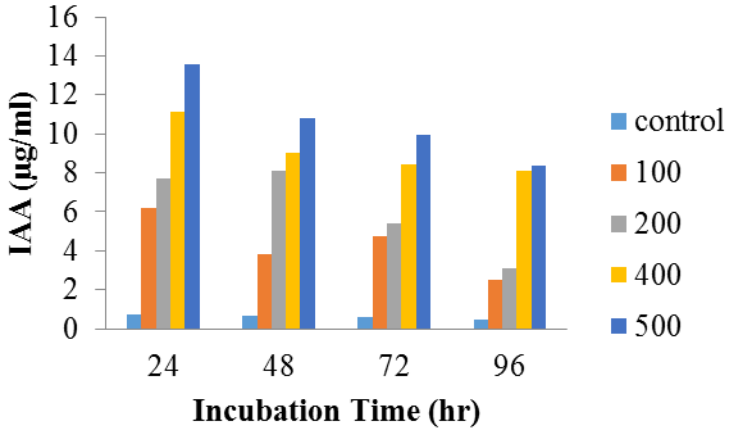


Fig.2 IAA production by *Nostoc muscorum* (CCC442) in presence of different levels of tryptophan (µg/ml)

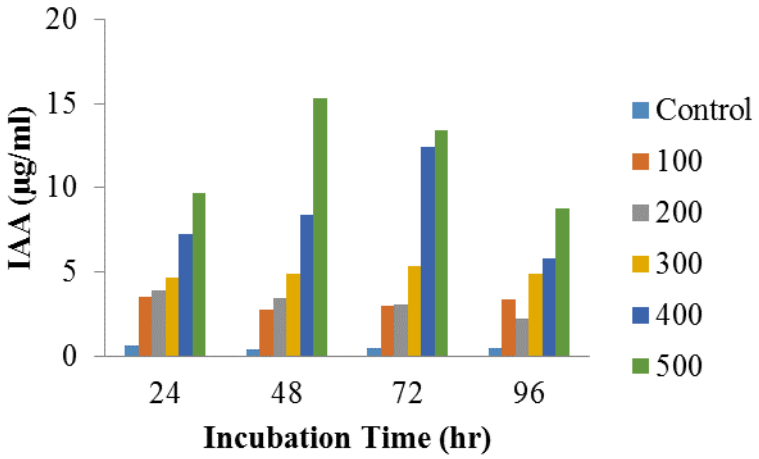
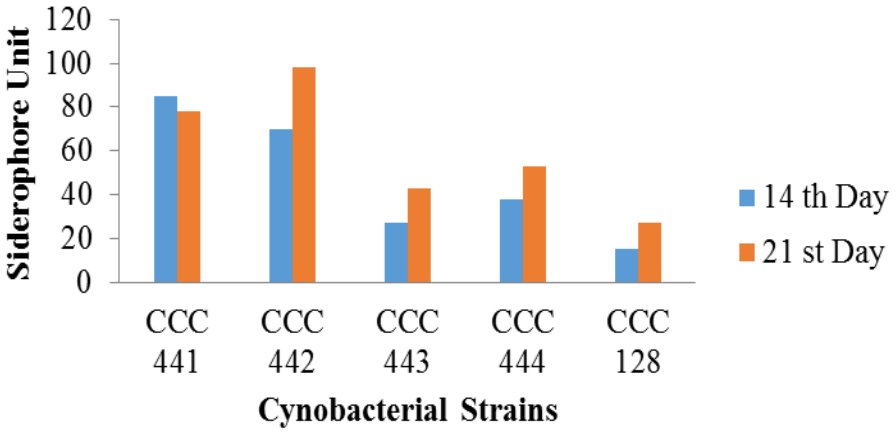


Fig.3 Siderophore production by cyanobacterial strains



Westelliopsis prolifica (CCC128) and *Aulosira fertilissima* (CCC444) which were on par statistically. *Tolypothrix tenuis* (CCC443) recorded minimum production of IAA. There was, however, negligible production of IAA without tryptophan i.e., control. In the present study, there was negligible amount of IAA synthesis in absence of tryptophan but all the organisms were able to synthesize and release IAA during 4 days growth cycle. The data has also indicated that IAA production increased with increasing level of tryptophan.

Siderophore production

Siderophore production in cyanobacterial strains was monitored in iron limited condition up to 21 days of incubation. Accumulation of siderophore increased with incubation time and was maximum at the end of the incubation period in most of the culture except *Anabaena variabilis* (CCC441) which showed maximum siderophore production at 14 days of incubation. The extent of siderophore accumulation in the growth medium was

culture dependent with *Nostoc muscorum* (CCC442) showing the maximum siderophore production along with *Anabaena variabilis* (CCC441) and closely followed by *Aulosira fertilissima* (CCC444) and *Tolypothrix tenuis* (CCC443). *Westelliopsis prolifica* (CCC128) showed minimum siderophore production (Figure 3). Cyanobacteria represent ubiquitous assemblages of photosynthetic prokaryotes which perform oxygenic photosynthesis and excrete a large number of organic and inorganic substances in medium in which they grow which are recognized as a key factor in plant growth promotion besides N_2 fixation (Lakshmi and Annamalai, 2008; Singh and Trehan, 1973; Malliga *et al.*, 2002). Gupta and Shukla (1969) have shown that the increase in growth of rice associated with culture of *Phormidium* sp. varied as a function of the fraction, the concentration, and the culture form which the extract was obtained. Karthikeyan *et al.,* (2007) has also identified promising cyanobacterial strains viz. *Calothrix ghosei*, *Hapalosiphon intricatus* and *Nostoc* sp. having plant growth promoting ability as evaluated by seed germination tests of wheat and hydroponic experiments. There were limited attempts have been made in the past to study the plant growth promoting nature of extracellular products of cyanobacteria. In earlier studies, the use of cyanobacterial extracts has shown a positive effect on germination and increase in growth of rice. In the present study, the effect of cyanobacterial culture filtrates from log phase and late log phase has been evaluated on rice seed germination and growth. All parameters studied have proved the potential of cyanobacteria which besides increasing N_2 fertility also benefit plant by its ability of growth promotion. The evaluation of extracellular exudates of 5 cyanobacterial cultures on germination as well as growth of the rice seedlings have shown 100% positive effect on rice seed germination with varying degree of effect on growth of roots, shoot length of seedlings. Phytohormones which influence plant growth are known to produce by diverse array of microbes (Glick, 1995; Whipps, 2001). The beneficial effects of cyanobacteria as biofertilizers, especially for rice crop, have often been interpreted as a result of biologically active substances produced by these organisms during their growth and proliferation in soil (Misra and Kaushik, 1989; Karthikeyan *et al.,* 2009). Among the growth regulators gibberellins, auxins, ethylene, cytokinins, abscissic acid and jasmonic acid have been detected in cyanobacteria (Gupta and Agarwal, 1973; Gupta, 1983; Stirk *et al.,* 1996). IAA is known to be one of the most physiologically active members of auxin family of phytohormones and has a role in root initiation and elongation and a number of other processes concerned with the differentiation and proliferation of plant tissues (Bartel, 1997; Arshad and Frenkenberger, 1998). The ability of cyanobacteria to produce the phytohormone IAA has been demonstrated (Sergeera *et al.,* 2002). Prasanna and co-workers (Prasanna *et al.,* 2008b; Prasanna *et al.,* 2009a) demonstrated the release of IAA by several Anabaena strains, which may play a significant role in plant growth promotion. A few reports on their plant growth promoting activity in wheat crop are also available (Karthikeyan *et al.,* 2007; Prasanna *et al.,* 2008b; Prasanna *et al.,* 2009a, 2010). It is well known that tryptophan serves as a physiological precursor for biosynthesis of auxin in plants and microorganisms and addition of tryptophan always has a stimulating effect (Patten and Glick, 1996; Spaepen *et al.,* 2007). To date, most IAA producing microorganisms are known to utilize pathways involving tryptophan as a precursor/intermediate. The tryptophan

independent pathways, which is more common in plants is known to occur only in Azospirilla (Costacurta and Vanderleyden, 1995; Spaepen *et al.,* 2007). Published reports on IAA production in cyanobacteria are scarce (Sergeeva *et al.,* 2002) in which only the tryptophan dependent production via the indole-3- pyruvic acid pathway was suggested. However, some scientists also reported tryptophan independent pathway in cyanobacteria Anabaena (Prasanna *et al.,* 2009a).In the present study, there was negligible amount of IAA synthesis in absence of tryptophan and all the organisms were able to synthesize and release IAA several folds higher in the presence of tryptophan than control during 4 days growth cycle.The data has also indicated that IAA production increased with increasing level of tryptophan. The siderophore production in cyanobacterial strains was monitored in iron limited condition until the stationary phase of their growth cycle. The accumulation increased with increase in incubation days but the increase was more during the exponential phase i.e., from 14 to 21 days. The results are in agreement with the earlier studies by Umamaheswari *et al.,* (1997) who have studied the siderophore production in two species of *Nostoc*. Initiation and synthesis of siderophores by the selected culture in iron limited growth medium indicated that the siderophore system develops primarily as a response to iron starvation in cyanobacteria (Clarke *et al.,* 1987). The observed differences in the siderophore production appears to have reasons due to species and strain diversity in cyanobacteria which might contribute to the siderophore accumulation and transport system as suggested for bacteria (Neilands, 1966).

The studies have demonstrated the ability of cyanobacteria to produce IAA and siderophores *in vitro* besides their inherent property of nitrogen fixation. Although, a lot of diversity exists among this group of organisms for these parameters but *Nostoc* and *Anabaena* which performed fairly better considering the overall performance and might have potential for development as a plant growth promoting inoculants.

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