

Modulation of IAA Production in Cyanobacteria by Tryptophan and Light

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Abstract

Cyanobacteria represent less a investigated group of prokaryote, in terms of their plant growth promoting potential, especially in relation to the production of phytohormones. The present investigation was aimed towards analyzing growth kinetics, indole acetic acid (IAA) production and acetylene reduction activity (ARA) as an index of nitrogen fixation in two selected cyanobacterial strains belonging to the genus *Anabaena*, as influenced by tryptophan supplementation and light:dark conditions. Interesting observations were recorded in terms of enhancement of IAA production accompanied by protein and chlorophyll accumulation in the two cyanobacterial strains grown in media without tryptophan and incubated under light:dark or continuous light conditions. Colorimetric and chromatographic analyses supported the observations that tryptophan is not essential as a precursor for IAA biosynthesis in these cyanobacteria. Further study is in progress to identify genes involved in the tryptophan independent pathway for IAA biosynthesis.

Key words: chlorophyll, cyanobacteria, IAA, nitrogen fixation, plant growth, tryptophan

Introduction

The role of microorganisms as plant growth stimulators is widespread in nature, especially in relation to the production of phytohormones. Auxins represent a group of plant hormones that are implicated in the regulation of diverse biological processes including cell division, elongation, differentiation, root elongation and tropistic responses. Among the auxins, IAA (indole-3-acetic acid) is recognized as a key factor, which is directly beneficial for plants. IAA is unique in being implicated both directly in plant growth promotion/formation of symbiotic associations, as well as indirectly in pathogenesis; therefore its contribution to plant related physiological processes and microbiological activity in soil is highly complex (Spaepen *et al.*, 2007). The ability to form plant hormones is believed to be a major property of several rhizospheric, epiphytic and symbiotic microorganisms including cyanobacteria, besides members of the plant kingdom (Arshad and Frankenberger, 1998).

Cyanobacteria represent a ubiquitous assemblage of photosynthetic prokaryotes of biological significance in being the progenitors of chloroplast and constitute a direct evolutionary link between pro- and eukaryotes. They are known to liberate a wide array of extracellu-

lar substances *e.g.* plant growth regulators, vitamins, amino acids, sugars *etc.*, which have direct or indirect impact on plant growth and yields (Mandal *et al.*, 1999; Nayak *et al.*, 2004; Prasanna *et al.*, 2008a). Many bacteria and fungi possess the ability to synthesize auxins using several pathways which provides them with the potential to form tight associations with plants or live as endophytes, and colonize the rhizosphere and phyllosphere. Among such microorganisms, some are involved in plant pathogenesis, while others stimulate plant growth.

Indole-3-acetic acid formation *via* indole-3-pyruvic acid and indole-3-acetic aldehyde is found in the majority of microorganisms, including phytopathogenic bacteria, such *Erwinia herbicola*, saprophytic species of genera such as *Agrobacterium*, *Pseudomonas* and *Azospirillum*, methylobacteria, symbiotic cyanobacteria-*Nostoc* sp., yeast *Saccharomyces uvarum* and phytopathogenic fungi such as *Fusarium*, *Rhizoctonia*, *Colletotrichum* sp. Alternative pathways include those in which tryptamine is formed (Kameneva and Muronets, 1999) or *via* indole-3-acetamide formation as in *Agrobacterium tumefaciens* and symbiotic bacteria such as *Rhizobium* sp. (Costacurta and Vanderleyden, 1995) or acetonitrile as an intermediate (Kameneva and Muronets, 1999). Interestingly, IAA

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biosynthesis is also known *via* tryptophan independent as well as dependent pathways in *Azospirillum* (Costacurta and Vanderleyden, 1995). Sergeeva *et al.* (2002) reported that cyanobacteria have the capacity to accumulate IAA endogenously and to release the hormone. The addition of tryptophan was found to stimulate the accumulation and release of IAA, indicative of a tryptophan dependent production of IAA. In our earlier studies, several strains of *Anabaena* (Prasanna *et al.*, 2008c) and cyanobacterial isolates from rhizosphere (Prasanna *et al.*, 2009a) exhibited the ability to excrete (mostly in the range of 1–3 µg/ml) in the nitrogen free BG-11 medium without added tryptophan. The comparison of IAA produced by bacteria *vis a vis* cyanobacteria seems quite unsuitable, as their cell volumes and mode of nutrition are highly different. However, studies have shown that bacterial strains can produce 3–55 µg ml⁻¹ of IAA (Patten and Glick, 1996; Zaidi *et al.*, 2006).

The objective of our investigation was to investigate the possibility of enhancing IAA production by modulating environmental factors, thereby, enhancing their potential for use as plant growth promoting agents. Hence, the current study focused on modulating IAA production in two cyanobacterial strains, with emphasis on the role of tryptophan as an inducer /precursor and its relationship with light:dark conditions.

Experimental

Materials and Methods

Organisms and Growth conditions. Preliminary screening undertaken with a set of *Anabaena* strains (Nayak *et al.*, 2009) and rhizosphere isolates of diverse

rice/wheat varieties (Karthikeyan *et al.*, 2007; Nayak *et al.*, 2007; Jaiswal *et al.*, 2008a; Prasanna *et al.*, 2009a), which had revealed their potential for IAA production and plant growth promotion in rice and wheat crop. A set of selected *Anabaena* strains screened in experiments undertaken in earlier studies (Prasanna *et al.*, 2009a) were grown and maintained in nitrogen-free BG-11 medium, without tryptophan (Stanier *et al.*, 1971) at 27±2° C under a light intensity of 52–55 µmol photon m⁻² s⁻¹ and 16L:8D (light:dark cycles; referred to as L:D in text, tables and figures).

Seed germination and growth assay. Late log phase (21 days) of the selected strains were centrifuged and the cell free filtrates were used for inhibition studies with wheat seeds (*var.* HD 2687) which had been sterilized using standard protocols (Shende *et al.*, 1977). After soaking for 36 h, the seeds were placed on seedling agar (1% water agar) and kept at 37°C in dark for germination after 48 h; percent of germination and length of coleptile and radicle were measured and tabulated.

Experimental set up. On the basis of performance of cell free filtrates on seed germination/growth assay, two strains (*Anabaena* sp. CW1 and *Anabaena* sp. RP9) were selected. These two strains were subjected to the following treatments: light/dark conditions – [continuous light (CL), light:dark cycles (16L:8D) and complete darkness (CD)] in the presence/absence of tryptophan (5 mg ml⁻¹) in the medium. Samples were taken periodically at the intervals of 7, 14 and 21 days and analyzed for various biochemical attributes. The growth of the cultures in terms of chlorophyll accumulation was estimated following the hot extraction method of MacKinney (1941) using methanol as the extracting solvent. Quantitative estimation of total

Tests of between-subjects effects dependent variable: IAA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
STRAIN	21.356	1	21.356	70.726	0.000
CONDITIO	248.067	2	124.034	410.760	0.000
DAYS	58.676	2	29.338	97.158	0.000
SUBSTRAT	0.318	1	0.318	1.054	0.308
STRAIN* CONDITIO	87.385	2	43.692	144.695	0.000
STRAIN * DAYS	73.376	2	36.688	121.499	0.000
STRAIN * SUBSTRAT	34.835	1	34.835	115.361	0.000
CONDITIO * DAYS	102.458	4	25.615	84.827	0.000
CONDITIO * SUBSTRAT	18.274	2	9.137	30.259	0.000
DAYS * SUBSTRAT	69.556	2	34.778	115.173	0.000
STRAIN * CONDITIO * DAYS	12.646	4	3.161	10.470	0.000
STRAIN * CONDITIO * SUBSTRAT	55.621	2	27.810	92.099	0.000
STRAIN * DAYS * SUBSTRAT	4.693	2	2.347	7.771	0.001
STRAIN * CONDITIO * DAYS * SUBSTRAT	107.525	8	13.441	44.511	0.000

All values were statistically significant at 5 percent level

soluble proteins was done spectrophotometrically using the protocol of Herbert *et al.* (1971) against bovine serum albumin as a standard. The amount of IAA was quantified spectrophotometrically by measuring the intensity of pink color at 530 nm using calibration curve of standard IAA stock solution (10–100 $\mu\text{g ml}^{-1}$) prepared in 50% ethanol (Gordon and Weber, 1951).

Measurement of acetylene reduction activity (ARA). Gas chromatographic quantification of ethylene formed was utilized as an index of nitrogen fixation. Commercially available ethylene was utilized for quantification and vials with an equivalent volume of water served as controls (Hardy *et al.*, 1973). Measurement of acetylene reduction activity (ARA) was done after 7, 14 and 21 days of incubation. Acetylene (10% v/v) was injected after removal of equivalent amount of air and incubation was done in light for 90 min. The ARA values were expressed as nmoles ethylene produced per ml culture. All the measurements were taken in triplicate.

Qualitative analysis using thin layer chromatography (TLC). To identify IAA, a standard solution (1 mg ml^{-1}) was spotted along with cell free filtrate (20 μl) of the cyanobacterial cultures from the various treatments on silica gel plates (Silica gel 60, Merck) and placed in solvent system comprising chloroform: acetic acid: 95:5 (Pillay and Mehdi, 1968). After 3 h, the plates were sprayed with Van Urk's reagent (1 g 4-dimethyl amino benzaldehyde dissolved in 50 ml diluted HCl 1:19) and R_f values compared with that of standard IAA.

Statistical analysis. The data, recorded in triplicates for the growth and biochemical parameters in selected two strains were subjected to ANOVA (Analysis of variance) in accordance with the experimental design (completely randomized block design) using SPSS.10 statistical package to quantify and evaluate the source of variation. Standard deviations (SD) are depicted in the graphs and critical differences (C.D.) values were calculated at a probability of 0.05, as given in Tables.

Results

Evaluation of the cell free filtrates of a set of seven cyanobacterial strains, belonging to the genus *Anabaena*, in terms of their effect on germination of wheat seeds was undertaken along with controls (Table I). Percent of germination ranged from 97–99%, with CW1, C11 and C16 recording 99% as compared to 97 and 98% in the controls treatments (BG 11 medium and sterile water). In terms of length of coleoptile and radicle recorded after 48 h, seeds soaked with cell free filtrates of RP9, CW1 and C6 strains showed significantly higher values (Table I). Earlier studies

Table I
Effect of extracellular filtrate of selected cyanobacterial cultures on seed germination and biometric parameters

Treatments	Length of Coleoptile (cm)	Length of Radicle (cm)	Percent of Germination (%)
Sterile water	1.020	3.214	98
BG-11	0.750	1.741	97
CW1	1.980	5.799	99
C6	1.710	5.173	98
C11	1.470	4.310	99
C16	1.030	3.147	99
C20	1.340	4.157	96
RP8	1.430	4.152	98
RP9	2.090	4.831	98
SEM	0.078	0.129	–
C.D.	0.164	0.270	–

(Prasanna *et al.*, 2008c; Prasanna *et al.*, 2009a) undertaken in terms of production of plant growth promoting metabolites by a set of *Anabaena* strains had also revealed that CW1 and RP9 as promising isolates. Hence, further studies were undertaken with RP9 and CW1 strains, by evaluating the interplay between conditions of illumination and supplementation with/without tryptophan on growth attributes and IAA production, as a prelude to their utilization as plant growth promoting inoculants for wheat crop.

Time course studies undertaken revealed an enhancement of chlorophyll in strain CW1 from 7 to 14 days with highest values being recorded under light:dark (16:8) conditions, in the absence of tryptophan (Fig. 1). The strain RP9 did not show any significant increase in chlorophyll under L:D conditions, although incubation under continuous light conditions, in the absence of tryptophan, enhanced chlorophyll accumulation over the entire period of incubation. Under continuous light incubation, RP9 strain recorded higher values as compared to CW1. Very low values were recorded in continuous dark conditions, in the presence/absence of tryptophan.

Anabaena sp. CW1 showed an enhancement in terms of IAA production from 7–14 days, under both continuous light and L:D conditions, in the absence of tryptophan. A highest value of 11.43 $\mu\text{g ml}^{-1}$ was recorded in 21 days cultures of *Anabaena* sp. CW1, in the absence of tryptophan supplementation and continuous light conditions. Two folds enhancement from 7–14 days was observed in strain RP9 under continuous light conditions in media with/without tryptophan, which remained static thereafter (Fig. 2). The strain RP9 showed an almost 3 folds enhancement by 14 days of incubation which remained static up to 21 days under continuous light conditions in the presence or absence of tryptophan. Under L:D

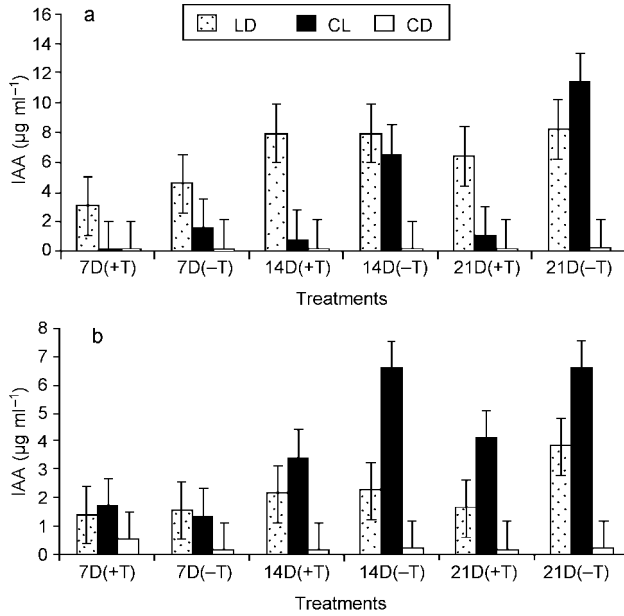


Fig. 1. Influence of tryptophan supplementation and light: dark treatments in chlorophyll accumulation after 7, 14 and 21 days in a. *Anabaena sp. CW1*; b. *Anabaena sp. RP9* incubated under light: dark cycles (L:D), continuous light (CL) and complete darkness (CD); +T and -T indicate the presence or absence of tryptophan.

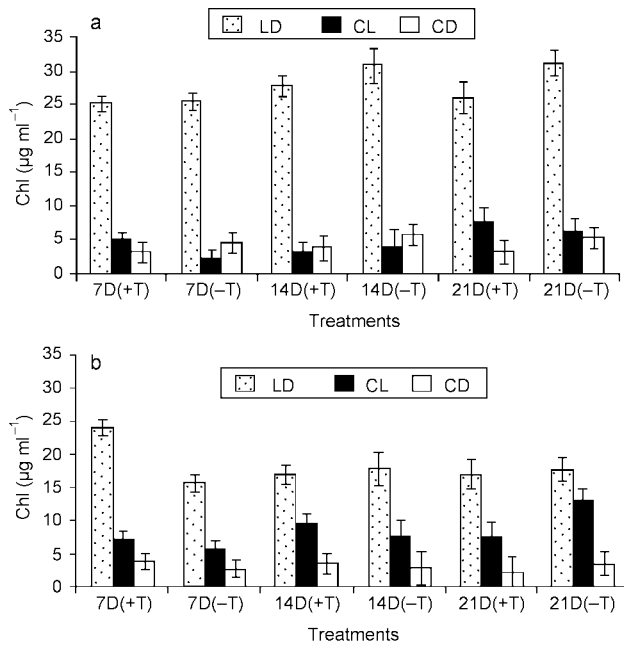


Fig. 2. Effect of tryptophan supplementation and light:dark treatments on IAA production in a. *Anabaena sp. CW1*; b. *Anabaena sp. RP9* incubated under light:dark cycles (L:D), continuous light (CL) and complete darkness (CD); +T and -T indicate the presence or absence of tryptophan.

conditions, a gradual enhancement in IAA production was observed over the period of incubation in the absence of tryptophan. Extracellular IAA under continuous darkness was less than 10% of the values recorded in illuminated conditions. Further evaluation was undertaken using thin layer chromatography

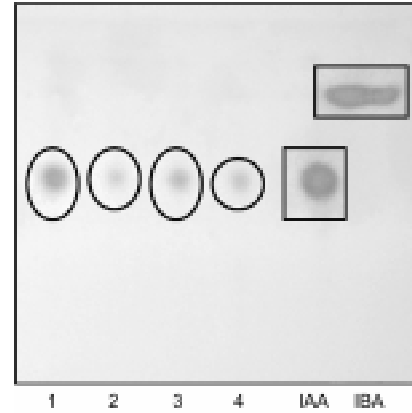


Fig. 3. Thin layer chromatographic plate showing the production of IAA in cyanobacterial strains under different light regimes. [1, 2, 3, 4, denote samples taken from *Anabaena sp. CW1* and *RP9*, incubated under continuous light and light-dark cycles respectively and IAA and IBA denote standards of indole-3-acetic acid and indole-3-butyric acid].

(Fig. 3) which revealed spots representative of IAA (based on R_f values of standard IAA) in all samples incubated under L:D or continuous light, illustrative of production of IAA by the cultures. No spots were observed in continuous dark incubated cultures.

Protein accumulation in *Anabaena sp. CW1* showed an enhancement under continuous illumination, in the presence of tryptophan, with highest values recorded in 21 days cultures, in the absence of tryptophan. No uniform trend could be observed in *Anabaena sp. RP9* and values were much lower than the other strain; the highest values were recorded for this strain in 7 days cultures under L:D conditions (Fig. 4).

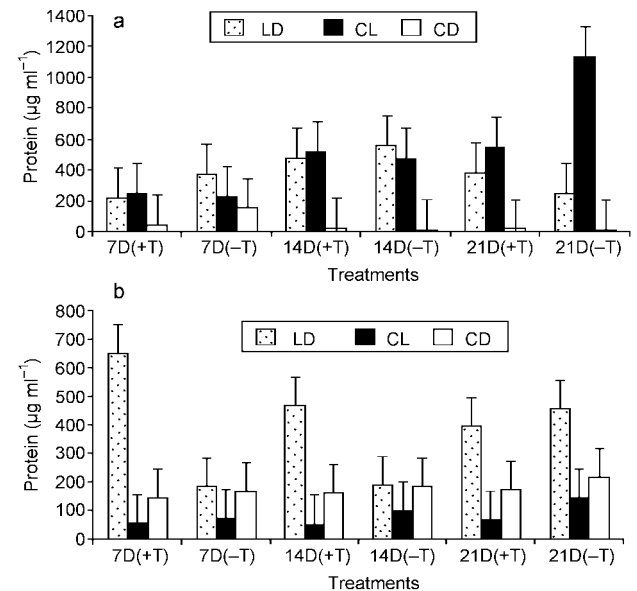


Fig. 4. Effect of tryptophan supplementation and light:dark treatments on protein accumulation after 7, 14 and 21 days of incubation in a. *Anabaena sp. CW1*; b. *Anabaena sp. RP9* incubated under light:dark cycles (L:D), continuous light (CL) and complete darkness (CD); +T and -T indicate the presence or absence of tryptophan.

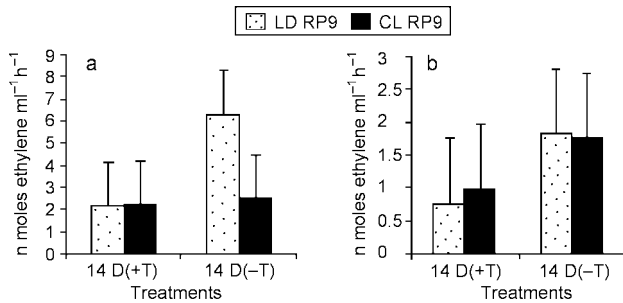


Fig. 5. Effect of tryptophan supplementation and light:dark treatments on nitrogen fixation (measured as acetylene reducing activity) after 14 days of incubation in a. *Anabaena* sp. CW1; b. *Anabaena* sp. RP9 incubated under light:dark cycles (L:D) and continuous light (CL) respectively; +T and -T indicates the presence or absence of tryptophan.

Acetylene reducing activity (as an index of nitrogenase activity/nitrogen fixation) of the cultures revealed that highest values were recorded after 14 days of incubation in light:dark (16:8) conditions (Fig. 5). In general, in the absence of tryptophan, both the cultures recorded higher values under L:D condition. No ARA was recorded in dark incubated cultures (data not shown).

Discussion

Plant associated microorganisms are generally capable of synthesizing phytohormones, which perform the role as mediators in signaling between plant and microflora. Among them, the genera *Azospirillum*, *Pseudomonas*, *Enterobacter*, *Gluconacetobacter* and *Rhizobium* have been investigated in terms of the physiological and genetic aspects related to IAA production (Tsavkelova *et al.*, 2006). Although IAA is known to be one of the most physiologically active auxins, involved in plant growth promotion and plant pathogen interactions, the biosynthesis and regulation of IAA has not been unraveled completely and strain specific differences have been recorded. Recent reports also suggest that IAA can be a signaling molecule in bacteria and can also, therefore, have a direct effect on bacterial physiology (Spaepen *et al.*, 2007; Srinivas *et al.*, 2002). Production of IAA under different light conditions undertaken in a set of anoxygenic phototrophic bacteria indicated a significant enhancement in the presence of tryptophan and illumination, rather than dark conditions (Srinivas *et al.*, 2002); however, in yeasts and fungi, dark conditions are known to enhance IAA biosynthesis (Yurekli *et al.*, 2003).

Cyanobacteria play diverse roles in the environment, as nutrient supplements (inoculants) and soil compaction agents in agriculture, besides having tremendous ecological significance as carbon sequestering and bio remediating agents (Venkataraman, 1975;

Kaushik, 2002; Prasanna *et al.*, 2008a). In recent years, they are being explored as sources of bioactive molecules having pharmaceutical and agricultural significance (Kulik, 1995; Jaiswal *et al.*, 2008b; Prasanna *et al.*, 2010). 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). A number of cyanobacterial strains have been reported to produce extracellular amino acids such as aspartic acid, glutamic acid, aspartic acid, proline, valine, glycine and alanine (Venkataraman and Saxena, 1963; Singh and Trehan, 1973; Karthikeyan *et al.*, 2007) at various stages of growth of the culture. Besides amino acids, extracellular polysaccharides such as xylose, galactose, fructose and several others have been reported to be excreted in the external medium of cyanobacteria. Liberation of vitamin B₁₂ has been reported from many other cyanobacterial strains (Misra and Kaushik, 1989). 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). Cyanobacterial extracts are known to promote somatic embryogenesis and organogenesis (Wake *et al.*, 1992; Bapat *et al.*, 1996; Manickavelu *et al.*, 2006). The production of phytohormone IAA by several free-living and symbiotically competent cyanobacterial strains was documented (Sergeeva *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). However, information on the kinetics of IAA production by these prokaryotes is limited (Sergeeva *et al.*, 2002; Prasanna *et al.*, 2008c).

The set of cyanobacterial strains being evaluated included isolates from soil samples from rice-wheat cropping sequence and rhizosphere of rice and wheat plants (Prasanna *et al.*, 2009a; Karthikeyan *et al.*, 2007). As a first step, the effect of cell-free filtrates of a set of seven strains on germination of wheat seeds was undertaken. The percent germination, length of radicle and plumule after 48 h revealed the potential of *Anabaena* sp. RP9 and *Anabaena* sp. CW1 which were then examined for the production of IAA as influenced by light:dark conditions and supplementation with tryptophan. Interestingly, higher chlorophyll, protein and IAA production was recorded in both the strains without tryptophan supplementation and incubated under L:D or continuous light conditions.

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). In our investigation, the evidence for production was further correlated with the TLC data, where spots were observed at R_f values corresponding to IAA.

The nitrogen fixing potential of both strains was measured after 7, 14, 21 days (data not shown). However, the highest values were recorded after 14 days in the tryptophan deficient and supplemented media under both conditions of illumination. Camerini *et al.* (2008) have reported an enhancement in acetylene reducing activity in rhizobia exhibiting overproduction of IAA. However, in cyanobacteria, the presence of tryptophan is known to induce several abnormalities in relation to heterocyst development and nitrogen fixation (Mishra and Tiwari, 1986). It supports our conclusions that tryptophan supplementation does not have a positive effect on nitrogen fixation. Comparatively, ARA was higher in tryptophan deficient cultures; in our study which emphasizes that BG-11 medium (devoid of tryptophan) is most suitable for the growth, nitrogen fixation and IAA production of the two cyanobacterial strains being investigated.

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 however, only the tryptophan dependent production *via* the indole-3-pyruvic acid pathway was suggested. Our results however, conclusively show that the production of IAA in the two cyanobacteria – *Anabaena* sp. CW1 and RP9 is several folds higher in the absence of tryptophan, and the growth attributes (chlorophyll, proteins) are also much higher under this condition. Therefore, it can be hypothesised that some cyanobacteria may also additionally have an tryptophan independent pathway for IAA, which is the first report in the case of cyanobacteria.

A balanced interplay of different factors, including bacterial IAA biosynthesis, rather than IAA production *per se* is needed to stimulate plant growth. The two strains used in our study were from diverse geographical locations. *Anabaena* sp. CW1 is a rhizosphere isolate from rice variety grown in Aduthurai (located in the state of Tamil Nadu, India) while RP9 is an isolate from soil samples from Pusa, Bihar (India). Earlier studies with *Anabaena* sp. CW1 had shown its potential for IAA production (Prasanna *et al.*, 2009a), but in-depth analyses regarding optimal conditions for

maximising IAA production was not undertaken. The current investigation clearly reveals that this strain can be a promising strain, as it can produce IAA, even in the absence of tryptophan in the environment and its inclusion in biofertilizer inocula will not only enhance plant growth but also stimulate microbial activity in the soil/rhizosphere, besides aiding in colonization. The second cyanobacterium utilized in this study has shown potential for production of IAA and hydrolytic enzymes in our earlier investigation (Prasanna *et al.*, 2008c), which is further strengthened by the observations in this study. Such strains can provide a continuous nutritional stimulation throughout the period of crop growth, which can help to overcome several problems related to their colonization and establishment. Further work, is in progress to elucidate the genes involved as well as the role of IAA in microorganism-plant interactions, for the effective utilization of these strains as inoculants for wheat crop.

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