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 extracellular 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, methylbacteria, 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 tumificiens and symbiotic bacteria such as Rhizobium sp. (Costacurta and Vanderleyden, 1995) or acetonitrile as an intermediate (Kameneva and Munorons, 1999). Interestingly, IAA
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}

<table>
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<th>Source</th>
<th>Type III Sum of Squares</th>
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<td>13.441</td>
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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 µg 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) 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 Rf 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 (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 µg 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 of tryptophan, which remained static thereafter (Fig. 2).

#### Table I

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Length of Coleoptile (cm)</th>
<th>Length of Radicle (cm)</th>
<th>Percent of Germination (%)</th>
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<tr>
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<tr>
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</tr>
<tr>
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<td>99</td>
</tr>
<tr>
<td>C6</td>
<td>1.710</td>
<td>5.173</td>
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<td>C11</td>
<td>1.470</td>
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<td>C16</td>
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<td>C20</td>
<td>1.340</td>
<td>4.157</td>
<td>96</td>
</tr>
<tr>
<td>RP8</td>
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<td>4.152</td>
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<tr>
<td>RP9</td>
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<tr>
<td>SEM</td>
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<td>0.129</td>
<td>–</td>
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<tr>
<td>C.D.</td>
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<td>–</td>
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</table>
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) 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).
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*, *Glucanacetobacter* 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 anoxicogenic photoautotrophic 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 B_{12} has been reported from many other cyanobacterial strains (Misra and Kaushik, 1989). Among the growth regulators gibberellins, auxins, ethylene, cytokinins, abscisic 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 Rf 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 micro-organism-plant interactions, for the effective utilization of these strains as inoculants for wheat crop.

Acknowledgements
The authors are grateful to the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi (AMAAS Network — after Indian Council of Agricultural Research for providing the facilities and financial support, in the form of projects for carrying out the present investigation.

Literature


