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Growth and biochemical characterization of associations between cyanobionts and wheat seedlings in co-culturing experiments

Anjuli Sood[1], Pawan K. Singh[2], Arun Kumar[3], Rajendra Singh[3] & Radha Prasanna[4]*

[1]*Department of Botany, University of Delhi, Delhi-110007, India, e-mail: anjulisood@gmail.com*
[2]*Department of Botany, Banaras Hindu University, Varanasi-221001, U.P., India*
[3]*National Phytotron Facility, Indian Agricultural Research Institute, New Delhi-110012, India*
[4]*Division of Microbiology, Indian Agricultural Research Institute, New Delhi-110012, India, e-mail:radhapr@gmail.com*

Abstract: N_2-fixing cyanobacteria are unique in their capacity to form symbiotic associations with a wide range of eukaryotic hosts belonging to different plant groups. The present study was undertaken to analyze the interactions of the cyanobiont PI 01 (from *Azolla pinnata*) and *Nostoc* PCC 9229 (from *Gunnera monoika*) with wheat seedlings, in co-culturing experiments. Each of the cyanobionts enhanced significantly the volume of root and shoot biomass in the experimental cultures. The transverse sections of roots in the co-cultured seedlings revealed the presence of aseriate packets of cyanobionts below the root epidermis. The investigated cyanobionts excreted amino acids (His, Met, Val) and sugars into the medium, while indoleacetic acid was detected when the cyanobionts were grown in a tryptophan containing medium. During the co-culturing, sugars and proline were detected in the extracellular filtrates. It can be hypothesized that these sugars and amino acids may serve as signal substances in the development of functional associations between the relevant cyanobionts and the wheat seedlings.

Key words: co-culturing; cyanobacteria; cyanobiont; symbiotic associations; tryptophan; wheat

Introduction

Cyanobacteria are a diverse group of gram-negative, photoautotrophic diazotrophs that occur both in free-living and symbiotic state. Some of them have the remarkable ability to form symbiotic associations with an extended range of eukaryotic hosts belonging to different plant groups i.e. bryophytes, pteridophytes, gymnosperms and angiosperms (Rai 1990; Rasmussen & Johansson 2002; Pabby et al. 2004a). However, loose associations between free-living cyanobacteria and plants are widespread in natural habitats (Whitton et al. 1988). Their occurrence and diversity in the rhizosphere of rice and wheat have been investigated (Jaiswal et al. 2008; Prasanna et al. 2009). Attempts in the past have been made to form adapted associations between crop plants and nitrogen fixing cyanobacteria with the intention to increase the contribution of biological nitrogen fixation in agriculture (Gantar 2000; Karthikeyan et al. 2009; Jaiswal et al. 2008). Several studies on the association between free-living/soil cyanobacteria such as *Synechococcus, Anabaena, Chlorogloeopsis, Nostoc* and cell/callus/protoplast cultures of *Panax, Medicago, Nicotiana* or the cuttings of rice, corn, bean, sugarbeet and wheat have been undertaken (Gantar 2000; Gusev et al. 2002). Despite availability of preliminary reports on associations between wheat roots and free-living cyanobacteria (Gantar et al.1995), information on characterization of associations with symbiotic forms is scanty. Preliminary investigations under controlled conditions from our laboratory have shown that cyanobacteria co-cultured with wheat bring about significant enhancement in soil microbiological and plant parameters (Karthikeyan et al. 2007; Jaiswal et al. 2008).

Bioactive substances such as amino acids, vitamins, phytohormones are known to be produced by free-living cyanobacteria or other diazotrophic bacteria under *in vitro* growth conditions (Gonzalez-Lopez et al. 2005; Gorelova 2006). They play an active role in the interactions with the host plants. In this context, cyanobacteria isolated from plant symbioses (in the following referred to as cyanobionts) could prove better choice than free-living strains in forming adapted associations (Rai et al. 1996). Several reasons have been put forward in support, which include (a) the ability of cyanobionts to adhere to the surface of organs and infect the crop plant; (b) capacity for reproduction on the surface or inside plant organs (c) fixation of atmospheric nitrogen and transfer most of the fixed nitrogen to the host (d) ability to use plant metabolites products for growth and nitrogen fixation (e) growth under heterotrophic conditions/microaerobic dark conditions (Johansson & Bergman 1994; Rasmussen & Johansson 2002; Sood et al. 2007). Previous studies have also

* Corresponding author

shown that *Nostoc* PCC 9229 were able to infect *Gunnera* by producing hormogonia, which is important for the establishing process resulting in the relevant symbiosis (Johansson & Bergman 1994). In this study hydroponic experiments were undertaken to analyze plant growth parameters and the chemical nature of the active compounds involved in the colonization process between the cyanobionts and wheat seedlings.

Material and methods

Cultivation of cyanobacteria

The axenic cultures of *Nostoc* PCC 9229 (symbiont isolated from *Gunnera monoika* which was grown under laboratory conditions in BG 11 medium) was kindly provided by Prof. U. Rasmussen (Stockholm University, Sweden), and a putative cyanobiont (isolated from *A. pinnata*; PI 01) were maintained as described earlier (Pabby et al. 2004b). The cyanobiont from PI 01 had chosen exhibited distinct morphological, physiological and molecular attributes as compared to its freshly separated counterpart (obligate symbiont) and cyanobionts from other species of *Azolla* (Pabby et al. 2003; 2004b; Sood et al. 2008). Both the cyanobionts were grown in chemically defined BG11 liquid medium without supplementation with $NaNO_3$ (Stanier et al. 1971) at a light intensity of 50–52 µmol photon $m^{-2}s^{-1}$, temperature of 25 ± 2 ℃ and photoperiod 16:8 h (Stanier et al. 1971).

Batch culturing of cyanobionts in the presence of tryptophan

The axenic cultures of cyanobionts of PI 01 (from *A. pinnata*) and *G. monoika* (*Nostoc* PCC 9229) were grown in nitrogen-free BG11 medium without and with filter sterilized tryptophan (500 mg L^{-1}) which acts as a precursor for indoleacetic acid (IAA) biosynthesis. All the cultures were subjected to Light:Dark (L:D, 16:8) or continuous darkness. 0.25 mL (packet cell volume) of log phase cultures of cyanobionts was taken and incubated to 250 mL flasks containing 100 mL of nitrogen free BG 11 medium in all the treatments. Analyses of growth parameters of cyanobionts (in terms of chlorophyll and proteins) and extracellular compounds were carried out after 15 d. All the experiments were repeated three times.

Co-culturing of wheat seedlings with cyanobionts under hydroponic conditions

Seeds of wheat (*Triticum aestivum* var. HD 2687*)* were surface sterilized with 0.1 % mercuric chloride for 30 min and thereafter washed thoroughly with sterile water. They were imbibed overnight in sterile water in dark at room temperature and kept for germination on seedling agar (2% agar in distilled water) in dark at 25 ± 2 ℃. The seedlings (4 d old) were placed in eppendorf tubes that had their tips removed to permit root growth. These were fixed in thermocol plates that were fitted to the top of pots (4") containing 300 mL of BG11 medium supplemented (with 0.5 g L^{-1} $NaNO_3$ to sustain threshold levels of nitrogen) for the growth of wheat seedlings for 20 d. The pots containing wheat seedlings in BG 11 medium (+ 0.5 g L^{-1} $NaNO_3$) served as control. A set of pots containing only the cyanobiont(s) (PI 01 and *Nostoc* PCC9229) was also maintained under similar conditions. Each pot contained 8 seedlings and treatments were taken in triplicate. 2 mL culture (packed cell volume) of each cyanobiont (approximately 3–5 µg mL^{-1} of chlorophyll *a*) was inoculated to each pot. All the pots were kept under controlled conditions of the National Phytotron Facility, IARI, New Delhi. This facility provides optimal temperature conditions (24 ± 2 ℃ at day and 20 ± 2 ℃ at night) for wheat throughout the growth period. After 20 d, the plants were harvested to assess the degree of association of cyanobionts with roots of seedlings under microscope and evaluate their effect on selected plant parameters. The culture medium was filtered through Whatman filter paper No. 1 and concentrated by lyophilization to 25% of original volume (Christ L-1, Alpha 2–41 model Lyophilizer) to analyze the probable nature of compounds released during co-culturing of cyanobionts with wheat seedlings and controls.

Analytical parameters

Chlorophyll *a* of the cyanobionts and the roots associated cyanobionts was estimated by the procedure of MacKinney (1941) after extraction in methanol. Protein content was measured by employing the procedure of Herbert et al. (1971) using bovine serum albumin as standard. The amount of extracellular sugars was determined by the methodology of Spiro (1966). The culture filtrate was used for estimation of auxin-like substances using Salkowski reagent (Gordon & Weber 1951). The filtrate was subjected to UV-Visible scanning from 200–1100 nm wavelength range to analyze the presence of unknown compounds. Acetylene reduction activity (ARA) was determined using gas chromatographic technique employing the method of Jewell & Kulasooriya (1970).

After 20 d of co-culturing, seedlings were harvested and their root length, shoot biomass and shoot chlorophyll were estimated. The chlorophyll of shoot(s) was determined after extraction of chlorophyll in DMSO (Dimethyl sulfoxide) using the methodology of Daizy & Kohli (1991).

The sections of excised roots were cut using wax microtomy (Brandon et al. 1964) to analyze the extent of colonization before and after vigorous shaking on a magnetic stirrer for 15 min and images was captured using Nikon light microscope (Microphot-FX).

The culture filtrate of co-culturing and controls (cyanobionts and wheat seedlings alone) was concentrated and the procedure of Kerkut & Shapira (1968) was used to determine free amino acids employing thin layer chromatography.

Statistical analyses

All experiments were conducted using complete randomized design with five replicates. Data was subjected to analysis of variance (ANOVA). The results shown refer to mean ± standard deviation, with F value in Table 1. For other experiments, statistical significance of mean values was analyzed by t-test at $p < 0.05$.

Results

Batch culturing of cyanobionts in the presence of tryptophan

Significant differences ($p < 0.05\%$) were observed in chlorophyll *a* and protein content of both the cyanobionts grown with or without tryptophan under Light:Dark and complete Dark conditions (Table 1). The content of chlorophyll *a* in *A. pinnata* and *G. monoika* cyanobionts was highest (10.83 ± 0.21 µg mL^{-1} and 27.63 ± 0.33 µg mL^{-1} respectively) in L:D (+trp) (Light:Dark with tryptophan) conditions. Similarly, protein content was also observed to be 2 folds higher in the cyanobiont of *A. pinnata* grown in media

Table 1. Growth parameters of cyanobionts from *A. pinnata* and *G. monoika* after 15 d of incubation.

Strains	Parameters[a]	Treatments				F value (Interaction)
		Light:Dark		Dark		
		– trp	+ trp	– trp	+ trp	
Cyanobiont from *A. pinnata*	Chlorophyll *a*	8.48 ± 0.80	10.83 ± 0.21	2.06 ± 0.31	1.69 ± 0.16	14.97
	Proteins	280.8 ± 29.41	781.5 ± 7.99	112.66 ± 8.95	345.4 ± 12.30	95.65
	ARA	176.18 ± 6.31	46.67 ± 5.67	17.74 ± 0.20	7.71 ± 1.85	250.23
Nostoc PCC 9229 isolated from *G. monoika*	Chlorophyll *a*	17.98 ± 0.08	27.63 ± 0.33	11.31 ± 0.02	11.78 ± 0.45	81.82
	Proteins	605.86 ± 16.21	947.23 ± 2.28	404.13 ± 3.48	623.33 ± 14.04	35.11
	ARA	14.15 ± 0.36	3.74 ± 0.56	4.23 ± 0.28	2.08 ± 0.04	140.54

[a]Chlorophyll and proteins as μg mL^{-1}; ARA as μmol C_2H_4 mg Chl^{-1} h^{-1}

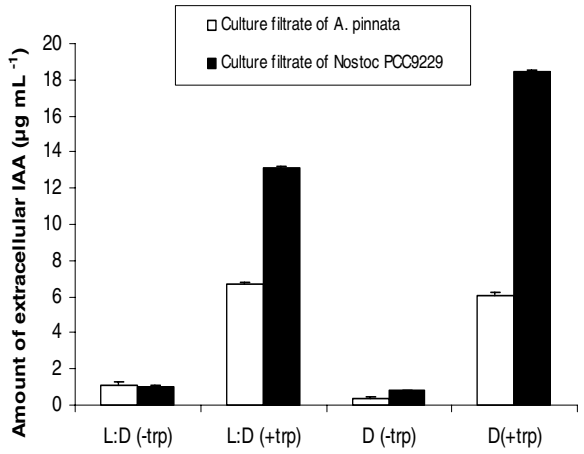

Fig. 1. Extracellular IAA (μg mL^{-1}) in the culture filtrates of the cyanobionts from *A. pinnata* and *Nostoc* PCC 9229 grown with or without tryptophan under L:D and complete darkness.

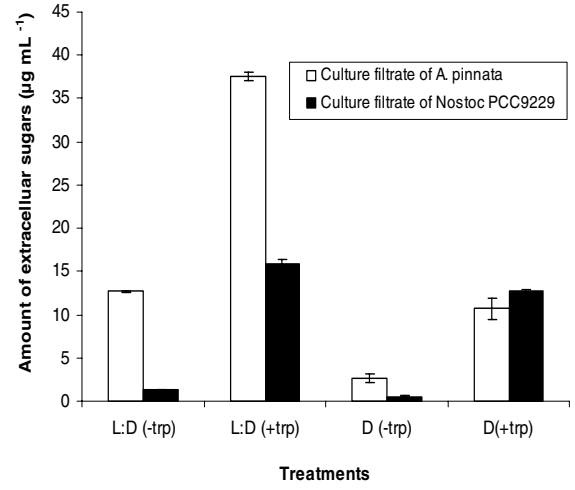

Fig. 2. Extracellular sugars (μg mL^{-1}) in the culture filtrates of the cyanobionts from *A. pinnata* and *Nostoc* PCC 9229 grown with or without tryptophan under L:D and complete darkness.

containing 500 mg mL^{-1} tryptophan under L:D conditions. On the other hand, the presence of tryptophan drastically suppressed acetylene reduction ability in the two cyanobionts, irrespective the settings of L:D and complete darkness. Considering the light/dark conditions, complete darkness had an inhibitory effect on the chlorophyll *a* and acetylene reducing activity, irrespective of the presence or absence of tryptophan (Table 1). However, the protein content of the two cyanobionts was significantly higher in tryptophan supplemented cultures, under both set of environmental conditions. The values were 2 folds higher in the cyanobionts of *A. pinnata* (PI 01), and 50% higher in *G. monoika* (*Nostoc* PCC 9229) in tryptophan supplemented cultures kept under complete darkness as compared to L:D treatments (Table 1).

The amount of extracellular IAA in the filtrate was higher in the cyanobionts incubated in tryptophan, irrespective of L: D and complete darkness (Fig. 1). Maximum amount of extra cellular sugars were recorded in both the cyanobionts grown under L: D (+trp) (Fig. 2). The UV-Visible scanning of culture filtrate showed peaks at 296 nm in filtrate of cyanobionts from *A. pinnata,* and 302–306 nm in filtrate of symbiotic *Nostoc* PCC 9229 isolated from *Gunnera* grown under L:D (+trp) and D (+trp) (complete darkness with tryptophan). However, no peak was observed in filtrate of both the cyanobionts grown under L:D (-trp) (light:dark without tryptophan) and D (-trp) (complete darkness without tryptophan).

Co-culturing of wheat seedlings with cyanobionts

The wheat seedlings co-cultured with the cyanobionts exhibited higher number of leaves/seedling as compared to the control (seedlings grown without cyanobionts) (Fig. 3a). Cyanobionts from *A. pinnata* (PI 01) as well as *G. monoika* (*Nostoc* PCC 9229) produced hormogonia within 36 h of co-culturing, and also formed associations with the roots of wheat seedlings (Fig. 3b, c). Longitudinal sections of wheat roots showed colonization behind the root cap area (Fig. 3e). In transverse sections, aseriate packets comprising filaments of cyanobionts were observed inside the cortical cells of roots of co-cultured wheat seedlings (Fig. 3d). The root length of co-cultured wheat seedlings with cyanobionts from *A. pinnata* (Wh + PI 01) and *G. monoika* (Wh + *Nostoc* PCC 9229) was significantly increased as compared to their control plants (Table 2). Similarly, the shoot biomass and chlorophyll of co-cultured wheat

Table 2. Effect of co-culturing of wheat seedlings with cyanobionts of *A. pinnata* and *G. monoika* (*Nostoc* PCC 9229) on plant parameters after 20d of growth.

Treatment	Root length (cm)	Shoot biomass (mg)	Total chlorophyll ($\mu g\ mL^{-1}$)	Total chlorophyll (μg mg fresh biomass^{-1})
Wheat (+1/3N)	25.33 ± 1.53	78.66 ± 3.43	14.67 ± 0.61	0.18 ± 0.04
Wheat + cyanobiont from *A. pinnata*	39.16 ± 0.76*	142.9 ± 24.10*	24.05 ± 2.54*	0.17 ± 0.02
Wheat + *Nostoc* PCC 9229	29.00 ± 2.65*	144.46 ± 30.91*	21.87 ± 1.58*	0.15 ± 0.03

* Significant at $p < 0.05$

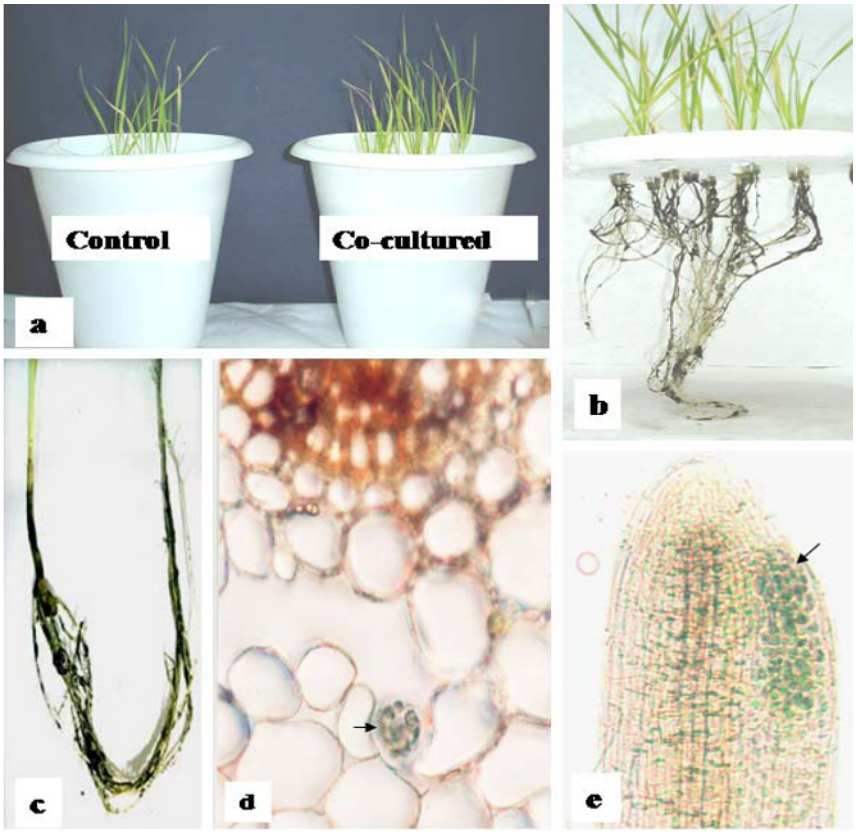

Fig. 3. a – experimental set up of co-culturing of cyanobionts with wheat seedlings; b – co-cultured wheat seedling with roots, showing association with cyanobiont; c – magnified view of root showing association with cyanobiont; d – T.S. of root showing the presence of cyanobionts below epidermis (f); e – L.S. of root showing the inntracellular presence of cyanobionts, below the epidermis.

seedlings were significantly larger than control (Table 2). However, no significant differences were observed in the total chlorophyll of the shoots+leaves, measured in terms of mg fresh biomass in the cyanobiont associated seedlings (Table 2). No measurable chlorophyll was recorded in the roots of wheat seedlings, grown without cyanobionts. The amount of chlorophyll *a* was highest in the cyanobionts growing alone, as compared to those of the roots-associated cyanobionts (Fig. 4). The acetylene reducing ability expressed as nmol C_2H_4 per vial was significantly higher in root-associated PI 01 cyanobiont, than in their free living counterparts, whereas change in ARA was non-significant ($p<0.05\%$) in case of *Nostoc* PCC 9229 (Fig. 4).

Thin layer chromatography of amino acids of concentrated filtrates of the cyanobionts showed the presence of three and two spots respectively, whose Rf values corresponding with those of standard Met, Val, His (PI 01) and Met, His (*Nostoc* PCC 9229) respectively. On the other hand, the filtrate of the cyanobionts co-cultured wheat seedlings showed one yellow coloured spot, which matched with proline. No amino acids were detected in the filtrate of wheat seedlings grown without cyanobionts. The spectrophotometeric analysis of the culture filtrates of cyanobionts alone and co-cultured wheat-cyanobiont revealed the presence of sugars (Table 3). However, the amount of sugars was much higher in the filtrates of the cyanobionts grown without wheat seedlings.

Discussion

Establishing adapted symbiosis between agriculturally important crop plants and nitrogen fixing microorganisms has been a much investigated topic, as a means of reducing the dependence on chemical nitrogen fertilizers (Gantar 2000; Gorelova 2006). Despite the availability of reports on this objective, findings have not been

Table 3. Extracellular sugars in culture filtrates of wheat-cyanobiont co-cultured seedlings after 20d.

Treatment	Extracellular sugars (μg mL^{-1})
Wheat seedlings	0.023 ± 0.01
Cyanobionts from *A. pinnata*	1.43 ± 0.02*
Wheat seedlings + cyanobiont from *A. pinnata*	0.66 ± 0.01*
Cyanobionts of *Nostoc* PCC 9229	3.13 ± 0.02*
Wheat seedlings + *Nostoc* PCC 9229	0.95 ± 0.13*

* Significant at $p < 0.05$

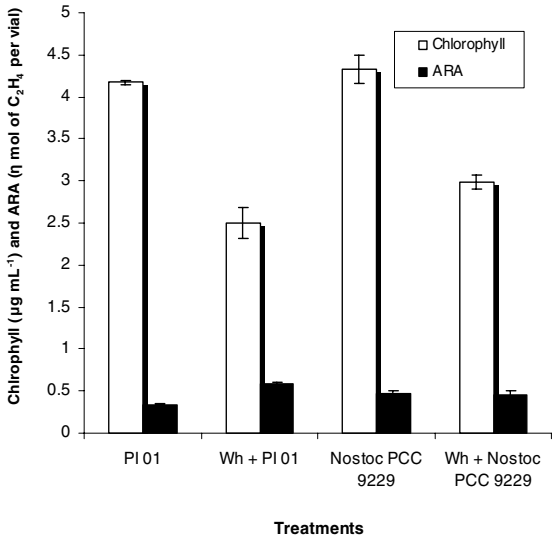

Fig. 4. Chlorophyll (μg mL^{-1}) and ARA (Acetylene Reduction Activity; measured as nmol of C_2H_4 per vial) of cyanobacteria and root-associated cyanobionts.

extended beyond laboratory level (Gantar 2000). This can be attributed to the paucity of information regarding cyanobacterial strains which can form functional associations (intra/inter cellularly). Wheat, along with rice occupies around 75% of total cultivated area globally and consumes a major share of the chemical nitrogenous fertilizers. It is well established knowledge that organisms with affinity towards roots, or residents of the rhizosphere can prove better competitors in soil, when used as inoculants. Therefore, for our studies we selected two cyanobacteria/cyanobionts which had been originally isolated from associations with *Azolla pinnata* (PI 01) and *Gunnera monoika (Nostoc* PCC 9229). Our investigation was aimed towards evaluating the associations formed between the relevant cyanobionts and wheat seedlings by recording of the plant growth promotion and the chemical compounds involved.

The communication between plant and microorganisms typically involves signal exchange, which is followed by the invasion of the microorganism and the accompanying structural changes in the plant. Gantar (2000) reported that filaments of *Nostoc* 2S9B penetrate both the root epidermis and cortex, forming packages in intercellular spaces. Previous investigations made in our laboratory have shown that short filaments/cells of *Calothrix ghosei* are present in the wheat root sections (Karthikeyan et al. 2009), while the rhizosphere isolates of cyanobacteria belonging to *Anabaena* and *Nostoc* exhibited inter/intracellular associations with wheat seedlings in co-culturing experiments (Jaiswal et al. 2008).

In the present study, the investigated cyanobionts produced hormogonia, which are considered as the first prerequisite for establishing associations under natural conditions (Adam et al. 2006). However, the exact mode of penetration of these motile hormogonia structures is not clear. Despite repeated shaking of the cyanobiont-associated roots, cyanobionts were observed both inter and intracellularly, indicating the nature of the association. Hydroponics, is a well established method for understanding plant-microbe interactions and provides a reliable and effective means of characterization of interactions between cyanobacteria and seedlings. In the present study, the water environment in the hydroponic study facilitated evaluation at all stages of co-culturing, in terms of both visual/microscopic and biochemical analyses. The nitrogenase activity (ARA) of the root-associated cyanobiont of *A. pinnata* was much higher than of the free-living cyanobionts used in this study. Almost 10 folds higher values were recorded than that obtained in our earlier investigation (Jaiswal et al. 2008). This can be attributed to the selected cyanobacteria used in this experiment, originally being symbiotic isolates, which have the ability to utilize carbon sources provided by the host as a source of reductant and energy. This may enable the cyanobacteria to sustain their nitrogenase activity even in dark. Earlier workers have reported that cyanobacteria utilize the root exudates as a source of energy during complete darkness, thereby maintaining their nitrogen fixing capacity (Gantar et al. 1995).

Phytohormones play an important role as signals that regulate growth and development in plants. Our results showed that the studied cyanobionts, tryptophan acts as precursor for the synthesis of IAA, followed by its excretion into their surrounding environments. This takes place both in the presence or absence of light, indicating that these cyanobionts possess genes for IAA biosynthesis (Sergeeva et al. 2002). The ability to synthesize plant hormones is believed to be a major property of rhizospheric, epiphytic and symbiotic bacteria/cyanobacteria that not only stimulate plant growth, but also play important role as signals in communication between plant host(s) and the relevant microflora (Kravechenko et al. 2004; Tsavkelova et al. 2006). In our investigation, cyanobionts retained their ability of IAA production under complete darkness. This may be due to their inherent symbiotic nature giving ability to

grow inside the host tissues and utilize the tryptophan or indole derivatives produced by the host (Johansson & Bergman 1994; Pabby et al. 2004a). The presence of tryptophan stimulated the growth of the cyanobionts under L:D conditions, whereas the acetylene reducing activity of these cyanobionts was suppressed in media containing tryptophan, which is itself a nitrogen source. The root length of co-cultured wheat seedlings were also larger than compared with corresponding wheat seedlings grown alone. These results are in agreement with the findings of previous investigations involving free-living cyanobacteria-plant associations (Svircev et al. 1997), and being attributed to the production of plant growth hormones by cyanobionts (Sergeeva et al. 2002).

The present investigation showed that the culture filtrate of PI 01 and *Nostoc* PCC 9229 cyanobionts showed the presence of Met, Val, His and Met, His amino acids respectively, whereas in the culture filature of cyanobionts co-cultured with wheat, only proline was observed. Extracellular amino acids have been reported in a culture of many free-living cyanobacteria including *Calothrix brevissima, Anabaena cylindrica, Chlorogloea fritschii, Cylindrospermum* sp. (Whitton 1965; Karthikeyan et al., 2009). Although extracellular sugars were also detected in the filtrates, further studies are needed to analyze the type of sugars present. It is well established that limnic and marine algae excrete organic substances into their surrounding growth medium. A large number of such molecules e.g. flavonoids, phenolic compounds, sugars and amino acids have been detected in vicinity of symbiotic tissues ascribed as potential signal substances in natural symbiotic associations (Bergman et al. 1996). In the present study, sugars and amino acids were detected in the filtrate of cyanobionts, which indicate their possible role as signals. Arabinogalactan proteins (AGPs) are known to be released by many free-living cyanobacteria, and these substances are thought to have an important role in plant growth and development (Bergman et al. 1996). Graf and Ruby (1998) suggested that host-derived free amino acids as well as peptides or proteins support proliferation of symbiotic bacteria. Kraffczyk et al. (1984) also reported that maize root exudates consisted of 65% sugars, 33% organic acids and 2% amino acids.

Future research needs to be directed towards the detailed microanalyses of the filtrates from the co-culturing experiments using sensitive assay techniques to identify the quorum sensing molecules and signals for understanding their exact role in the development of functional associations between cyanobionts and wheat seedlings.

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