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Research Paper

Physicochemical confirmatory evidences for cyanobacterial released plant growth hormones governing escalation of rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) crop

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Abstract

Numerous plant-associated bacteria produce auxin and related indolic compounds. These indolic compounds were assessed based on their biological effects upon plants, such as shoot elongation and root hair deformation (Avena curvature test) or coleoptile elongation. Thus, precise identification of auxin and related molecules obtained following extraction of bacterial culture supernatants, concentration of the extracts, and separation and identification of the compounds by using thin layer chromatography (2D-TLC), high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry. The present study includes in-vitro screening of 31 cyanobacterial isolates from 11 different genera along with one reference culture having history of 5 to 6 years rice-wheat crop organic practices. The cyanobacterial isolates were tested for their physiological role in rice-wheat growth promotion with reference to seed germination, seedling root-shoot elongation and secretion of growth promoting substances followed by detection and chemical identification of growth related substances like Indole-3-acetic acid and Indole-3-butyric acid. Among these test isolates, the highest germination 94.67 (±00.52) per cent with vigor index 76.69 (±02.85) was observed in rice seeds treated with 21 days old intracellular exudates of *Westillopsis* sp. (CCC-554) and the rice seedling plumule length 12.63 (±01.050) cm was observed in rice seed treated with 21 days old intracellular exudates of isolate *Nostoc* sp. (CCC546) while the longest radical length 09.18 (±00.17) cm was observed in rice seed treated with 14 days old cellular exudates. The highest wheat seed germination 99.00 (±00.57) per cent with vigor index 2533(±96) was observed in case of *Chroococcus minutus* (CCC582) and the wheat seedling plumule length 16.13 (±00.52) cm was observed in seeds treated with 21 days old cellular exudates of isolate *Microcystis robusta* (CCC568), while the longest radical length 23.270 (±0.173) cm was observed in wheat seed treated with 21 days old cellular exudates of *Anabaena* spp. (CCC573). Cyanobacterial isolate *Cylindrospermum* sp. (CCC547) exhibited highest 27.53 (±01.332) µg per ml Indole-3-acetic acid (IAA) production during dark incubation period. Along with their growth promoting and regulatory effects the seletected isolates were subjected for chromatographic analysis using 2D TLC and HPLC for the presence of indolic substances in the different algal extracts which were under study. The separated spots of different cyanobacterial culture extracts of *Westelopsis* sp. (CCC559), *Nostoc* sp. (CCC560), *Chroococcus minutus* (CCC582) and the reference culture *Nostoc muscurum* (UTEX387) on thin layer chromatography (2D TLC) have comparable rate of flow to authentic Indole compounds like Indole-3-acetic acid and Indole butyric acid (IBA) where the retention time was similar to those of the authentic IAA and IBA

standard. The further confirmation of these results and identification of the endogenously produced indolic substances were achieved by high pressure liquid chromatography (HPLC) technique and GC-GC/MS.

Keywords: Cyanobacteria, Growth hormone IAA, Oryza, Triticum, Spectrophotometry, 2D TLC and HPLC

Introduction

Cyanobacteria are photosynthetic prokaryotes. Despite the fact they are often referred to as blue-green algae, they have no direct relation to higher algae. The agricultural importance of cyanobacteria in rice cultivation is related with their ability to fix nitrogen and other positive effects for plants and soil. The excessive use of chemical fertilizers has generated several environmental problems including the greenhouse effect, ozone layer depletion and acidification of water. In maintenance and build-up of soil fertility, consequently increasing rice and wheat growth and yield as a natural Cyanobacteria play an important role. These organisms also have potential in environmental management as soil conditioners, biofertilizers, biomonitors of soil fertility and water quality. They can be amelioratory agents in reclamation of saline and usar lands, and in rehabilitation of degraded ecosystems through biosorption of metals, feed for animals and protein supplement[1]. Also Some similar to auxins in their biological activity in culture filtrate of *Anabaena cylindrica* and *Oscillatoria* sp. concentrated culture filtrates of *Cvlindrospermum* sp. gave positive tests for gibberellin- like substances[2]. The indole-3-acetic acid (IAA), production by rhizobacteria is associated with plant growth promotion i.e. root initiation and elongation. Being Plant growth promoting (PGP) organisms, cyanobacteria can affect plant growth by different mechanisms viz. siderophore production[3,4]. The studies for the improvement of plant growth and seed production using *Nostoc* sp., *Anabaena* sp., *Phormidium* and *Spirulina* performed sp.[5] whereas some workers used *Oscillatoria* and *Westiellopsis* sp. in their experiments[6]. Similar studies has been carried out on cyanobacterial isolates for plant growth promoting activity by analyzing enhancement in rice-wheat seed germination, production of ammonia, IAA and phosphate solubilization[7]. Factors that affect the distribution of cyanobacteria include pH, soil moisture, mineral nutrients and combined nitrogen. Due to importance to this organism in present study we considered them worthwhile to examine the existence of cyanobacteria from rice-wheat crop field having history of organic cultivation and to analyze their growth parameters, physiological attributes and biochemical importance for their possible agronomic applications. Multiple cyanobacteria produce auxin-like compounds, such as the phytohormone indole-3-acetic acid (IAA), which is hypothesized to be important in the establishment of cyanobacterial associations with photosynthetic eukaryotes (Figure 2). In support of this, 83% of the symbiotic isolates tested positive for the production of auxin-like compounds compared to 38% of the free-living ones[3]. Moreover, IAA produced by *Nostoc* was recently shown to be necessary for it to colonize plant roots and additionally promoted plant growth[8,9]. These cyanobacteria are recognized to be prolific producers of bioactive compounds drawing interests as a source of various nutraceuticals, biomass and pigments[10].

Materials and Methods

Selection of isolates

Initially, thirty cyanobacterial strains isolated from organic farming soil having history of 5 to 6 years of organic farming practice of rice-wheat cropping system belonging to 11 different genus the four isolates were from *Cylindrospermum* sp. (CCC541, CCC547, and CCC567) including *Cylindrospermum musicola* (CCC552), eleven isolates from *Nostoc* sp. (CCC546, CCC553, CCC560, CCC571 and CCC575) including two from *Nostoc muscorum* (CCC548 and CCC555), one from *Nostoc punctiformae* (CCC551), one *Nostoc linkia* (CCC561), one *Nostoc spongiformae* (CCC563) and one reference strain *Nostoc muscurum* (UTEX387) along with seven isolates from *Anabaena* sp. (CCC550, CCC564, CCC570, CCC573, CCC574, CCC576 and CCC580), two isolates from *Westelopsis* sp. (CCC554 and CCC559) and each of isolate from *Anabaenopsis* sp. (CCC556), *Calothrix* sp. (CCC557), *Lyngbya perelegans* (CCC558), *Microcystis robusta* (CCC568), *Chroococcus minutus* (CCC582), *Phormidium* sp. (CCC543) and *Aphanothece* sp. (CCC549) were investigated along with one reference strain *Nostoc muscurum* (UTEX387) and were procured from the Culture Collection of Algae, University of Texas, Austin, USA was kindly provided by Dr. Alexy A. Vepritskiy (Darris Fresh Water Institute, RPI, Bolton Landing, NY, USA) and the seeds of rice (variety Pusa Sugandh *cv*. PUSA-2511) and wheat (variety Shreshtha *cv*. HD-2687) were procured from Centre for

Conservation and Utilisation of Blue Green Algae (CCUBGA), Division of Microbiology, IARI, New Delhi 110 012, India (Table 1).

Table 1: Cyanobacteria isolated from organic rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) organic field soil

S. No.	Isolate Code	Cyanobacterial isolates	S. No.	Isolate Code	Cyanobacterial isolates
1	CCC541	*Cylindrospermum* sp.	17	CCC560	*Nostoc* sp.
2	CCC543	*Phormidium* sp.	18	CCC561	*Nostoc linkia*
3	CCC546	*Nostoc* sp.	19	CCC563	*Nostoc spongiformae*
4	CCC547	*Cylindrospermum* sp.	20	CCC564	*Anabaena* sp.
5	CCC548	*Nostoc muscorum*	21	CCC567	*Cylindrospermum* sp.
6	CCC549	*Aphanothece* sp.	22	CCC568	*Microcystis robusta*
7	CCC550	*Anabaena* sp.	23	CCC570	*Anabaena* sp.
8	CCC551	*Nostoc punctiformae*	24	CCC571	*Nostoc* sp.
9	CCC552	*Cylindrospermum musicola*	25	CCC573	*Anabaena* sp.
10	CCC553	*Nostoc* sp.	26	CCC574	*Anabaena* sp.
11	CCC554	Westiellopsis sp.	27	CCC575	*Nostoc* sp.
12	CCC555	*Nostoc muscorum*	28	CCC576	*Anabaena* sp.
13	CCC556	*Anabaenopsis* sp.	29	CCC580	*Anabaena* sp.
14	CCC557	*Calothrix* sp.	30	CCC582	*Chroococcus minutus*
15	CCC558	*Lyngbya perelegans*	31	UTEX387*	*Nostoc muscurum*
16	CCC559	Westiellopsis sp.			

Note: *Reference strain, isolate no., 1 to 20 from rice organic field and Isolate 21 to 30 from wheat organic field

Growth and maintenance of cyanobacterial culture

These isolates were grown and maintained in BG-11 medium, with or without nitrogen under discontinuous illumination of 16hr:8hr light/dark cycle at 2500–3000 Lux light intensity using cool white fluorescent tubes at 28±2°C temperature in a culture room[11].

Assessment of rice-wheat plant growth promotion using crude algal extract

All the 31 cyanobacterial isolates were tested for their plant growth promotion attributes *viz*. rice-wheat seed germination, enhancement of radical and plumule length etc. using extra cellular and cellular culture exudates. The seeds of rice (variety Pusa Sugandh *cv*. PUSA-2511) and wheat (variety Shreshtha *cv*. HD-2687) were surface sterilized with 70 per cent ethanol and washed subsequently by two times with double distilled sterilized water and dried on autoclaved filter paper. The sterilized seeds were then placed in autoclaved petri plate containing sterile filter paper in one petri plate and then treated individually with 30 ml test cyanobacterial culture filtrate. The extra cellular (exogenous) culture filtrate was obtained by centrifuging 14 and 21 days old culture and the supernatant were used. The intra cellular (endogenous) exudates of 14 and 21 days old cyanobacterial culture was obtained with same pellet retained in case of extra cellular filtrate preparation. The culture pellet was washed two times with double distilled water and then subjected to macerate in mortar and pestle and sonication followed by centrifugation to obtain intracellular exudates as supernatant which was used for seed treatment. The control seed treatment was done with autoclaved double distilled water and BG11 medium with and without nitrogen source. The petri plates containing treated seeds were kept in incubator at 25±2^{0}C temperature. The culture filtrate was applied on seed at 24 hours interval in order to keep the treated seeds wet. The treated seeds were subjected for recording per cent seed germination up to 7 days after treatment. The panicle and radical length of germinated seedling was recorded at 14 and 21 days of interval. The experiment was carried out in triplicate and the mean value was taken[12].

Screening of cyanobacteria for Indole acetic acid production

The pink color develops when a mineral acid is added to a solution containing Indole acetic acid in the presence of ferric chloride. Different mineral acids, HCL, phosphoric acid, nitric acid, sulphuric acid and perchloric acid can be used for development of color. Since Beer's law is not followed at high concentrations of IAA, absorbance values obtained are converted to IAA concentration by a standard

curve. Such a curve does not vary appreciably with reagent stored in light at room temperature. All the 31 cyanobacterial cultures were screened for IAA production treated with and without different L-tryptophan concentration *viz*. 0, 50, 150, 300, 400 and 500 µg/ml in light and dark conditions. The cultures were grown at 28±2^{0}C for five days time interval in light and dark conditions separately. 2mL of culture supernatant was taken in 15mL test tube and 2 drops of Orthophosphoric acid was added along with prepared 4 mL of Salkowski reagent and the tubes were incubated for half an hour in dark by covering with aluminum foil to develop pink color. The absorbance was taken at 24 hours time interval at 530 nm. The IAA produced was quantified by preparing calibration curve of standard IAA (Indole Acetic Acid @10 to 100µg/ml). The experiment was carried out in triplicate and the mean value was taken[13,14].

Identification of growth promoting substances IAA and IBA

The stock solutions were prepared by dissolving 1 mg of each auxin (IAA and IBA) in 10 mL of methanol separately and also as a mixture, and subsequent dilutions were made with ratio of methanol to water 80:20 to prepare the desired concentrations. 5 mg algal biomass was dried, using an Operon bench top freeze dryer (FDB5503), and extraction was performed under the commonly used sonication conditions of 20°C for the optimized time of 30 min. The extraction solvents were methanol: water in a ratio of 80:20. The cyanobacterial test culture extracts of *Westelopsis* sp. (CCC559), *Nostoc* sp. (CCC560), *Chroococcus minutus* (CCC582) and the reference culture *Nostoc muscurum* (UTEX387) were centrifuged at 7500 rpm for 10 min, the supernatant was filtered through 0.45 µm syringe filter and concentrated to 500–1000 µL using an Organization NEVAP. The volume was made upto 50 ml by methanol and subjected to 2D TLC, HPLC and GC/MS analysis with different solvent phases. For GC/MS the rotary evaporated extract was dissolved in minimum amount of acetone and then the final volume was made upto 50 ml by hexane[15].

Qualitative analysis using thin layer chromatography (2D TLC)

IAA identification was done by preparing standard of Auxin solution (IAA & IBA 1mg per ml) and spotted along with cell free filtrate (20 µL) of the cyanobacterial culture from the various treatments on silica gel plates (Silica gel 60 Merck). The plates were placed in solvent system comprising chloroform: acetic acid (95:5). After 3 hours the plates were sprayed with Van Urk's reagent (1g 4dimethyl amino benzaldehyde dissolved in 50 ml diluted HCL 1:19) and R_f values compared with that of standard auxin (IAA and IBA)[15].

Preparation of samples

In present study, based on their effect on seed germination, root-shoot elongation and secretion of growth promoting substance like IAA, algal cultures were selected. The algal mass of selected isolates was dried and final weighed 5 gm sample was taken for further analysis. This 5 g sample was transferred into a 50 ml centrifuge tube. 5 ml HPLC-grade water was added to the centrifuge tube and vigorously vortexed, followed by addition of 10 ml of acetonitrile containing acetic acid 0.5%. The centrifuge tube was vortexed two times for 1 min using a vortex mixer at 15 min intervals. The centrifuge sample tube was then put into a refrigerator for 30 min to allow samples mixture to attain 4^{0}C. After the 4 g of Magnesium sulfate and 1 g of sodium chloride were added to the well-mixed algal mass/acetonitrile/water mixture and the mixture was vortexed immediately for 1 min. The sample was centrifuged for 5 min at 4000 rpm in 4°C using a refrigerated centrifuge. The acetonitrile extract (2 ml) was transferred to a 5 ml micro centrifuge tube containing 50 mg Primary secondary amine (PSA), 300 mg MgSO4 and 20 mg Graphite carbon black (GCB), followed by vortexing for 1 min. The 5mL micro centrifuge tube was centrifuged at 4000 rpm for 5 min in 4°C. The extract (1.2 ml) was transferred to a 1.8 ml Eppendorf vial and was reduced to nearly dryness using a vacuum concentrator. Acetonitrile (0.4 ml) containing 0.1 mgl^{-1} of triphenyl phosphate (TPP) was added to the Eppendorf vial. The vial was vortexed and centrifuged at 1000 rpm for 5 min. The extract was transferred to the HPLC and further to GC auto-sampler vial for GC–MS analysis[31].

Analysis by High performance liquid chromatography (HPLC)

For separation of analysts Merck Hitachi Eurosphere RP 18 (1005C_{18} column, 250 × 4mm) was used. The column was eluted with a linear gradient (0–5 min, 60 per cent A, 5–20 min, 100 per cent A) at a flow rate of 1mL per min of methanol (A) and 0.3per cent acetic acid (AcOH), and the column temperature was maintained at 25°C. Considering the ultraviolet (UV) maxima of auxin, UV detection was performed at λ max 225 nm and the excitation and emission wavelengths in the fluorescence detector were at 280 nm and 360 nm wave length respectively. The extracts of selected isolates *Westelopsis* sp. (CCC559), *Nostoc* sp. (CCC560), *Chroococcus minutus* (CCC582), the reference

culture *Nostoc muscurum* (UTEX387) and standards were injected (injection volume: 20 µL) into the reverse phase column and identifications were carried out using comparison of retention times and UV spectrums of the extracts with standard mixture. Each experiment was repeated at least three times and run in triplicate. Recoveries were calculated by adding a known amount of standards to the test cultures and extracting the auxin by the same method as described above[16]. For different samples analysis the HPLC conditions were same as above where samples taken after column fractioning purification after every 2 min, where subsequently each fraction was assayed in analytical column under the same conditions.

Results and Discussion

Plant growth promotion

Thirty one cyanobacterial strains isolated from organic rice-wheat crop field including with one reference cyanobacterial strain from *Nostoc muscurum* (UTEX387) were analyzed for their role in rice-wheat plant growth promotion and confirmation of growth regulatory substances viz. IAA and IBA. All the 31 cyanobacterial isolates belonging to 11 different genera were tested for cell free extract or extracellular exudates and intracellular exudates for the germination of rice and wheat seed (Plate 1). The results were briefed in terms of rice-wheat seed germination, vigor index, root-shoot development and their regulatory role.

Effect of extracellular culture exudates

Rice seeds (variety Pusa Sugandh *cv*. PUSA-2511) treated with cyanobacterial extracellular exudates with 14 days old culture of *Westiellopsis* sp. (CCC554) showed 81.33 (±3.93) per cent seed germination with vigor index 1008.67 (±21.21) followed by *Nostoc punctiformae* (CCC551), *Westiellopsis* sp. (CCC559) and *Nostoc spongiformae* (CCC563) which showed 72.00 (±4.04) per cent with varying vigor index 583.40 (±35.90), 729.30 (±63.46) and 525.08 (±15.47) respectively. Whereas seeds treated with 21 days old cyanobacterial extracellular exudates showed lower germination as compared to seeds treated with 14 days old culture exudates with low vigor index. The per cent wheat seed germination was recorded for the wheat seeds treated with 14 days extracellular exudates of isolate *Cylindrospermum* sp. (CCC567) was 86.67 (±1.333) per cent, for isolate *Anabaena* sp. (CCC570) was 86.00 (±1.155) per cent and for isolate *Anabaena* sp. (CCC574) was 88.00(±0.577) per cent, while the cultures with 21 days old extracellular exudates *Cylindrospermum* sp. (CCC567) showed 76.00 (±2.309) per cent, isolate *Anabaena* sp. (CCC570) showed 76.00 (±1.155) per cent as compared to control as a water which was 72.00 (±1.732) per cent which was higher side. The highest vigor index of germinated wheat seed was recorded in seed treated with 14 days old extracellular exudates and the isolate *Chroococcus minutus* (CCC582) showed 2354.06 (±76). The 21 days old cyanobacterial culture extracellular exudates of isolate *Microcystis robusta* (CCC568) showed vigor index of 3288 (±97) followed by isolate *Anabaena* sp. (CCC580) which was 2884 (±14) and isolate *Chroococcus minutus* (CCC582) was showed vigor index 2868(±15) (Figure 1).

The rice seedling plumule length was recorded for exponentially grown 14 days old cyanobacterial extracellular exudates was 7.43(**±**0.35) cm in isolate *Westelopsis* sp. (CCC554) followed by rice test seeds treated with culture exudates of *Cylindrospermum* sp. (CCC541) was 7.12 (±0.65) cm and *Anabaenopsis* sp. (CCC556) was 7.07 (**±**0.32) cm. On the other hand among the 21 day old cyanobacterial culture extracellular exudates tested on rice seeds the isolate *Nostoc* sp. (CCC546) showed 10.09 (**±**0.85) cm rice seedling plumule length followed by the isolate *Westelopsis* sp. (CCC554) which was 7.40 (**±**0.76) cm and that of isolate *Phormidium* sp. (CCC543) showed 7.00 (**±**0.12) cm successively. The wheat seedling plumule length was 8.467 (±0.186) cm in case of seeds treated with 14 days old extra cellular exudates of *Anabaena* sp. (CCC570). While the 21 days old extracellular exudates of *Anabaena* sp. (CCC576) showed 21.887 (±0.551) cm plumule length, (CCC568) (*Microcystis* sp.) showed 21.860 (±0.493) cm plumule length and *Anabaena* sp. (CCC580) showed 18.407 (±0.463) cm radical length. The short radical length 6.80 (**±**0.17) cm was observed in seeds treated with 14 days old extracellular exudates of *Nostoc* sp. (CCC561). Whereas the reference culture *Nostoc muscurum* (UTEX387) showed only 54(±07.10) per cent seed germination. The control treatments of cyanobacterial culture medium BG11 (+N), BG-11 (-N) and water showed average seed germination in the range of 39 (±02.00) to 42 (±08.01) per cent and the average plumule length 03.17 (±00.20) cm. The wheat seedlings treated with 21 days old extracellular exudates of cyanobacterial isolate *Anabaena* sp. (CCC580) and *Microcystis robusta* (CCC568) showed same 16.13 (±00.52) cm plumule length each (Table 2 and 3 and chart 1-4).

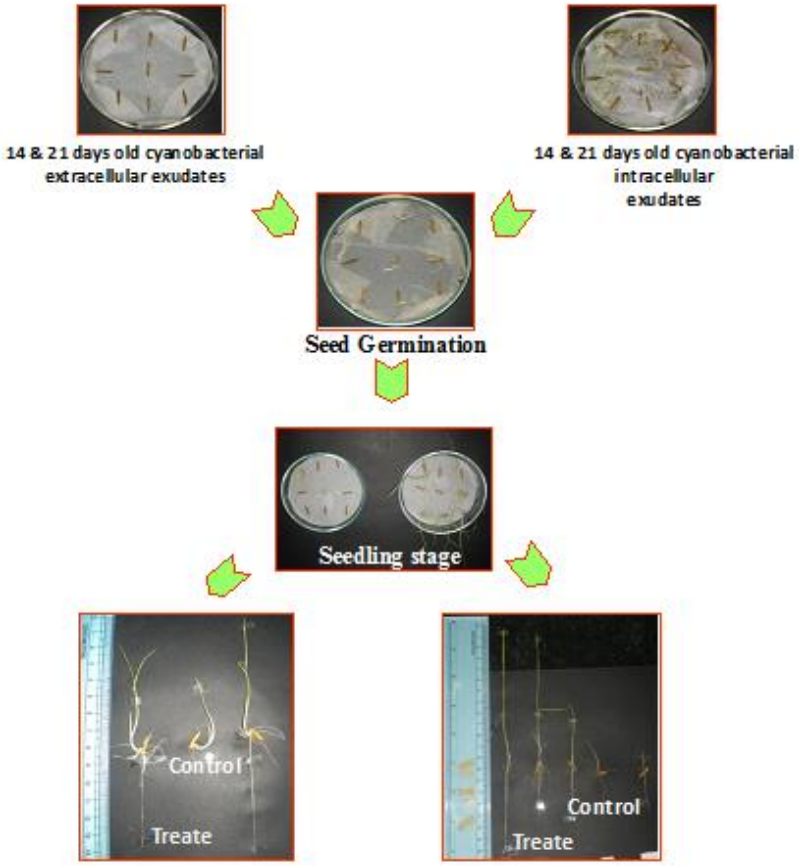

Figure 1: Effect of cyanobacterial intra and extra cellular exudates on seed germination, plumule and radicle length

Table 2: Effect of cyanobacterial culture exudates on rice seed germination (%)

S. No.	Culture code***	14 ECE*	14 ICE**	21 ECE*	21 ICE**
1.	CCC541	62.00±02.89 **(51.97)**	79.00±01.56 **(51.97)**	48.00±05.13 **(43.88)**	85.00±06.35 **(67.25)**
2.	CCC548	**72.00**±03.61 **(58.08)**	81.00±03.61 **(51.12)**	59.00±02.31 **(50.21)**	74.00±02.65 **(59.37)**
3.	CCC554	**81.33**±03.93 **(64.43)**	**89.33**±02.91 **(61.51)**	66.00±05.03 **(54.36)**	**94.67**±02.85 **(76.69)**
4.	CCC559	72.00±04.04 **(58.08)**	**91.00**±03.12 **(52.18)**	74.00±02.89 **(59.37)**	84.00±06.03 **(66.46)**
5	CCC563	**72.33**±03.76 **(58.29)**	88.33±05.51 **(58.34)**	**72.00**±01.73 **(58.08)**	83.67±03.53 **(66.20)**
6.	UTEX387	54.00±07.10 **(47.32)**	71.00±02.50 **(45.51)**	57.67±08.25 **(49.44)**	61.67±10.73 **(51.77)**
7.	WATER	42.67±08.01 **(40.80)**	41.67±02.23 **(40.31)**	50.33±03.22 **(45.23)**	41.67±05.11 **(40.40)**
8.	BG-11 (-N)	36.33±02.19 **(32.19)**	34.33±11.11 **(35.19)**	45.33±03.46 **(39.21)**	36.13±01.12 **(38.13)**
9.	BG-11 (+N)	39.00±02.00 **(38.67)**	36.00±02.03 **(32.14)**	40.00±02.18 **(39.25)**	39.00±02.00 **(38.24)**
CD at 5%		**10.33**	**11.52**	**11.14**	**17.19**

Arc sine value in bold letter, *ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates, ***refer table 1 for names assigned to culture code

Table 3: Effect of cyanobacterial culture exudates on wheat seed germination (%)

S. No.	Culture code***	Days after inoculation			
		14 Days ECE*	14 ICE Days**	21 ECE Days	21 ICE Days
1.	CCC567	86.67±01.33	96.00±00.57	76.00±02.309	92.00±01.73
		(79.89)	**(78.56)**	**(60.75)**	**(73.80)**
2.	CCC573	82.00±01.73	99.00±00.57	61.00±01.73	92.00±01.53
		(63.49)	**(78.56)**	**(48.47)**	**(78.30)**
3.	CCC582	89.00±00.57	99.00±00.57	72.00±01.15	96.00±00.44
		(85.42)	**(85.42)**	**(58.09)**	**(85.42)**
4.	UTEX387	81.00±01.73	97.00±00.57	60.00±01.73	90.00±01.53
		(71.70)	**(84.32)**	**(50.29)**	**(74.67)**
5.	Water	76.00±01.73	76.00±01.73	76.00±01.7	76.00±01.73
		(60.73)	**(60.73)**	**(60.73)**	**(60.73)**
6.	BG 11 (-N)	60.00±00.57	60.33±02.33	60.00±01.53	60.67±03.75
		(50.79)	**(51.00)**	**(50.80)**	**(51.22)**
7.	BG 11 (+N)	61.67±01.33	61.67±04.10	60.00±05.68	61.67±04.66
		(51.78)	**(51.82)**	**(50.86)**	**(51.83)**
(CD at 5%)		**04.783**	**05.056**	**05.348**	**03.96**

Arc sine value in bold letter, *ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates

Effect of intracellular culture exudates on rice-wheat seed

The rice seeds treated with 14 days old intracellular culture exudates of *Westiellopsis* sp. (CCC559) showed 91.00 (±04.04) per cent seed germination with 1198.47 (±36.45) vigor index, followed by isolate *Westiellopsis* sp. (CCC554), 89.33 (±03.93) percent with 1353.35 (±12.52)vigor index, while at 21 days it showed highest 94.67(±02.85) per cent seed germination. The per cent wheat seed germination in seeds treated with 14 days old cultures intracellular exudates of both *Anabaena* sp. (CCC573) and *Anabaena* sp. (CCC574) showed highest 99.00 (±0.577) followed by *Nostoc* sp. (CCC575) showed 97.33 (±0.666) while 21 days old culture isolate with intracellular exudates of isolate *Nostoc* sp. (CCC571) that showed 96.00±1.528, *Anabaena* sp. (CCC576) showed 96.00 (±0.577). The longest rice seedling plumule length was observed in rice seeds treated with isolate *Westelopsis* sp. (CCC554) of 14 days old cyanobacterial intracellular exudates was 9.13(**±**0.35) cm, followed by treatment with exudates of isolate *Anabaenopsis* sp. (CCC556) was 8.45 (**±**0.32) cm and that of isolate *Cylindrospermum* sp. (CCC541) intracellular exudates rice seed treatment showed 8.19 (**±**0.65) cm in decreasing order (Table 4 & Figure 2).

Table 4: Effect of cyanobacterial culture exudates on rice plumule length (Cm)

S. No.	Culture code***	14 ECE*	14 ICE**	21 ECE*	21 ICE**
01.	CCC541	07.12±00.65	08.19±00.65	06.80±00.92	08.00±00.40
02.	CCC554	07.43±00.35	09.13±00.35	07.40±00.76	07.20±00.31
03.	CCC556	07.07±00.32	08.45±00.32	06.90±00.71	10.92±01.16
04.	CCC560	06.37±01.00	07.54±01.00	06.87±00.70	11.93±00.84
05.	UTEX387	03.94±00.16	05.24±00.16	05.00±00.12	06.99±00.29
06.	WATER	03.79±00.55	03.60±00.55	03.60±00.40	03.50±00.35
07.	BG-11 (-N)	03.21±00.24	03.11±00.24	03.23±00.49	03.30±00.29
08.	BG-11 (+N)	03.17±00.20	03.27±00.20	03.10±00.17	03.33±00.62
	CD at 5%	**01.23**	**01.28**	**01.33**	**01.63**

***ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates, ***refer table no. 1 for names of culture code**

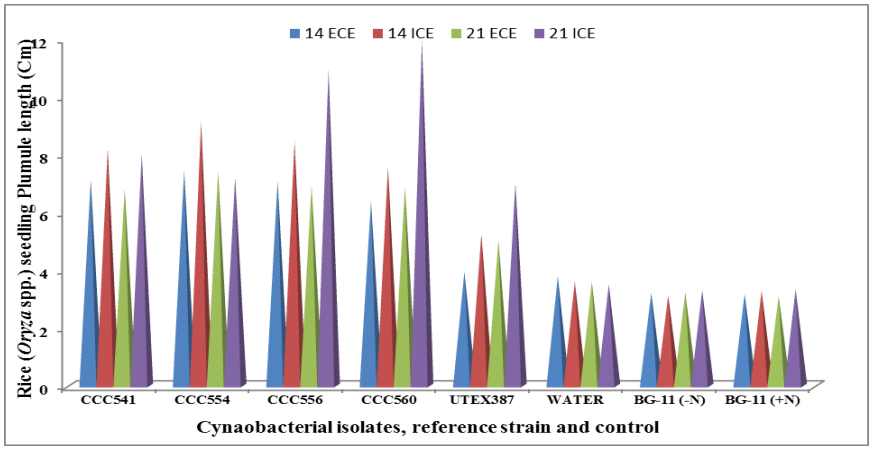

Figure 2: Effect of cyanobacterial culture exudates on rice plumule length (Cm)

The longest rice seedling radical length was observed with seed treatment of intracellular culture exudates in 14 day old culture isolate Nostoc linkia (CCC561) 09.18(±00.17) cm and *Calothrix* sp. (CCC557) was 08.90 (±00.23) cm as compared to reference culture (UTEX-387) was 05.21(±0.06) cm. The 21 day old culture extract treated rice seed showed radical length of 10.20 (±00.52) cm in isolate *Phormidium* sp. (CCC543), whereas the isolate *Nostoc muscorum* (CCC555) showed 09.00 (±00.17) as compared to reference culture *Nostoc muscurum* (UTEX-387) 03.10 (±00.12) cm (Table 5 & Figure 3).

Table 5: Effect of cyanobacterial culture exudates on rice radical length (Cm)

S. No.	Culture code***	14 ECE*	14 ICE**	21 ECE	21 ICE
2.	CCC543	06.30±00.58	07.20±00.58	09.20±00.55	10.20±00.52
10.	CCC553	03.60±00.29	04.70±00.29	04.20±00.58	09.00±00.17
12.	CCC555	03.00±00.06	04.00±00.06	03.50±00.12	09.00±00.17
13.	CCC556	04.20±00.17	05.86±00.17	05.00±00.17	08.00±00.35
21.	UTEX387	03.90±00.06	05.21±0.06	03.70±00.12	03.10±00.12
22.	WATER	04.60±00.46	05.10±0.46	04.50±00.06	04.50±00.35
23.	BG-11 (-N)	03.10±00.17	02.50±0.17	04.10±00.17	02.00±00.12
24.	BG-11 (+N)	03.00±00.23	03.00±0.23	03.10±00.17	02.90±00.12
	CD at 5%	**00.66**	**00.59**	**00.657**	**00.69**

***ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates, ***refer table no. 1 for names of culture**

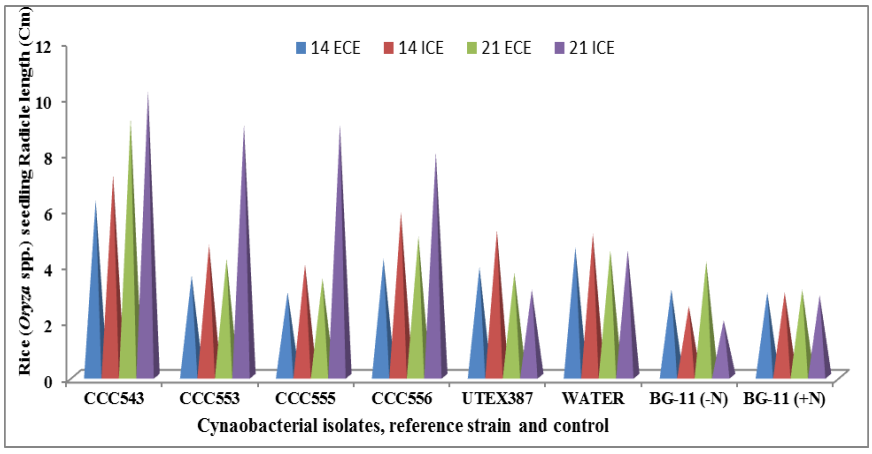

Figure 3: Effect of cyanobacterial culture exudates on rice radical length (Cm)

Wheat seeds treated with cyanobacterial exudates of isolate *Anabaena* sp. (CCC580) showed 14.10 (±0.549) cm plumule lengths. While the wheat seeds treated with 14 days intracellular exudates of isolate Nostoc sp. (CCC571) showed 12.960 (±0.000) cm long plumule length followed by 12.417 (±0.674) cm with cellular exudates of Anabaena sp. (CCC576). The better plumule length was observed in seedlings treated with 21 days old intracellular exudates of *Microcystis robusta* (CCC568) 16.13(±00.52) cm followed by *Chroococcus minutus* (CCC576) 14.00 (±0.75) cm (Table 6 and Figure 4).

Table 6: Effect of cyanobacterial culture exudates on wheat plumule length (Cm)

S. No.	Culture	Days after inoculation			
		14 Days ECE*	14 Days ICE**	21 Days ECE	21 Days ICE
1	CCC568	07.00±00.63	12.83±00.55	14.00±00.17	16.13±00.52
2	CCC576	07.05±00.09	12.42±00.67	11.00±00.17	14.00±00.75
3	CCC582	08.49±00.32	12.29±00.20	11.44±00.40	13.59±00.46
4	UTEX387	06.90±00.06	09.21±0.06	09.70±00.12	10.10±00.12
5	Water	10.66±00.49	10.42±01.48	11.07±02.09	10.99±01.17
6	BG 11 (-N)	10.04±00.60	10.05±00.13	10.38±01.01	10.00±00.16
7	BG 11 (+N)	11.70±00.75	10.03±00.97	11.03±00.56	10.37±00.64
CD at 5%		**01.293**	**02.096**	**01.551**	**01.844**

***ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates**

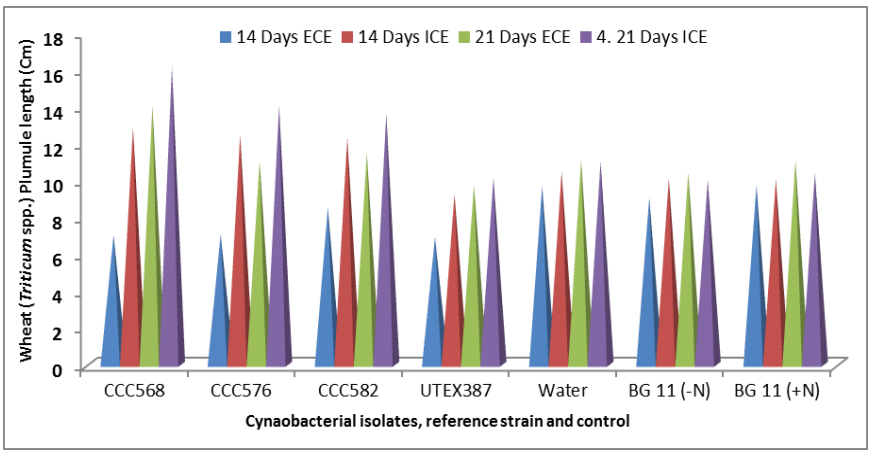

Figure 4: Effect of cyanobacterial culture exudates on wheat Plumule length (Cm)

The longest wheat seedling radical length intracellular culture exudates was 23.270(±0.173) cm observed with cyanobacterial culture *Anabaena* sp. (CCC573) followed by *Microcystis robusta* (CCC568) which was 22.130 (±0.551) cm and with *Chroococcus minutes* (CCC582) was 21.580 (±0.529) cm (Table 7& Figure 5).

Table 7: Effect of cyanobacterial culture exudates on wheat radical length (Cm)

S. No.	Culture code***	Days after inoculation			
		14 Days ECE*	14 Days ICE**	21 Days ECE	21 Days ICE
2.	CCC568	07.333±00.52	20.927±00.17	14.860±00.49	22.130±00.55
5.	CCC573	06.000±00.06	20.490±00.57	12.597±00.39	23.270±00.17
8.	CCC576	06.720±00.46	20.330±00.23	17.660±00.38	21.887±00.55
	UTEX387	08.120±00.43	18.820±00.40	15.060±00.50	17.010±00.21
11.	Water	15.380±00.35	15.017±01.70	16.003±01.01	18.693±01.41
12.	BG 11 (-N)	15.890±00.29	15.703±00.89	14.900±01.24	16.190±00.60
13.	BG 11 (+N)	17.263±00.52	17.280±00.53	17.293±00.52	16.943±00.82
CD at 5%		**01.255**	**03.071**	**01.687**	**02.082**

*ECE-Extra-cellular exudates, **ICE-Intra-cellular exudates

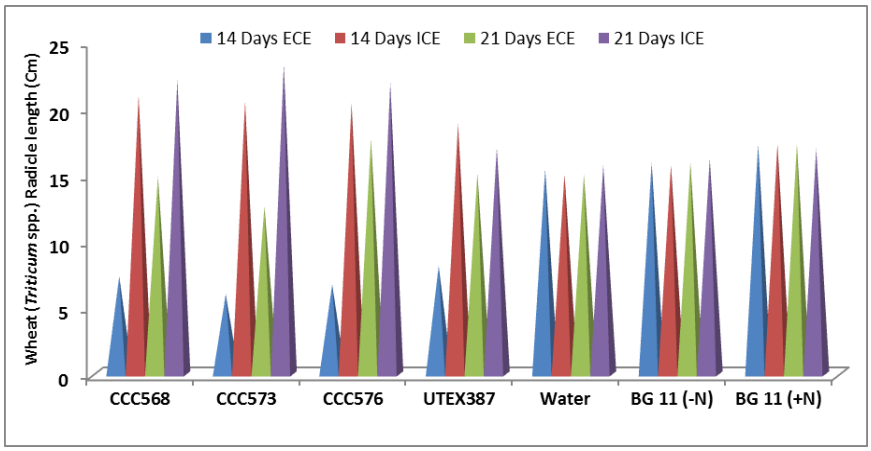

Figure 5: Effect of cyanobacterial culture exudates on wheat radicle length (Cm)

Spectrophometric estimation of Indole Acetic Acid Production

The Indole acetic acid production by cyanobacterial isolates was recorded at different days interval (light and dark) and tryptophan levels (0, 100, 200, 400 and 500 µg/ml).

Table 8: IAA production in light condition at different concentration of L-tryptophan (µg ml-1 culture)

S. No.	Culture code	Tryptophan concentration (µg/ml)				
		0	100	200	400	500
4.	CCC-547	01.22±00.11	03.70±00.16	07.43±00.38	16.03±01.29	19.76±00.38
8.	CCC-551	02.99±00.36	05.65±00.26	09.30±00.36	15.88±00.60	16.53±00.56
10.	CCC-553	01.19±00.14	04.82±00.25	07.10±00.51	15.13±00.70	16.16±00.78
31	UTEX-387	01.36±00.26	05.94±00.57	07.58±01.34	08.95±01.47	12.59±02.73
CD at 5%		01.63	01.85	02.31	02.27	02.53

At zero L-tryptophan concentration in day or light exposure the isolate *Nostoc punctiformae* (CCC-551) showed 02.99±00.36 IAA µg/ml of culture and isolate *Nostoc muscurum* (UTEX-387) showed 01.36±00.26 IAA µg ml^{1} of culture and isolate *Nostoc* sp. (CCC-553) showed 01.19±00.14 IAA µg ml of culture respectively 01.63CD at 5 per cent .While at 500 µg per ml L-tryptophan concentration the

rice field isolate *Cylindrospermum* sp. (CCC547) showed IAA production 19.76±00.38µg per ml of culture, the isolate *Nostoc punctiformae* (CCC551) showed 16.53±00.56 IAA µg/ml of culture and isolate *Nostoc* sp. (CCC553) showed production 16.16 (±0.78) IAA µg/ml of culture where as UTEX-387 showed 12.59±02.73 with 02.53CD at 5% (Table 8).

In dark condition at 0 (zero) L-tryptophan (µg/ml) concentration in *Nostoc* sp. (CCC560) it was 02.67 (±01.300) µg per ml of culture, while *Anabaena* sp. (CCC580) showed 02.05 (±00.123) µg ml^1 cultures and *Nostoc* muscurum (UTEX-387) showed 00.19±00.27. At 100 µg/ml L-tryptophan concentration isolate *Nostoc punctiformae* (CCC551) showed 5.60(±00.513) µg per ml of culture IAA production and isolate *Chroococcus minutus* (CCC582) showed 07.08 (±01.824) IAA µg ml^1 culture while reference culture *Nostoc muscurum* (UTEX387) showed IAA production 6.21 (±0.391) µg per ml of culture with 2.95 CD at 5 per cent. On the other hand at 500 µg per ml concentration of L-tryptophan isolate *Nostoc* sp. (CCC560) showed highest 17.35 (±01.20) IAA µg per ml of culture, also the isolate *Chroococcus minutus* (CCC582) showed 17.14±00.71 IAA µg per ml of culture reference culture *Nostoc muscurum* (UTEX387) showed 14.52±01.55 IAA µg per ml production with 03.38 CD at 5 per cent (Table 9).

Table 9: IAA production in dark condition at different concentration of L-tryptophan (µg ml^{-1} culture)

S. No.	Culture code	L-Tryptophan concentration (µg/ml)				
		0	100	200	400	500
17.	CCC-560	02.67±01.30	04.91±00.43	06.52±00.72	12.73±00.78	17.35±01.20
27.	CCC-575	01.42±00.08	04.63±00.34	06.21±00.58	14.06±01.11	16.96±01.29
30.	CCC-582	01.58±00.58	07.08±01.82	11.28±01.65	12.73±02.23	17.14±00.71
31.	UTEX-387	00.19±00.27	06.21±00.39	09.06±00.80	11.22±01.36	14.52±01.55
	CD at 5%	00.62	02.19	02.95	03.41	03.38

Identification of IAA

Based on the results of bioassay, it was suggested that the growth effect of crude algal extracts might be due to the presence of gibberellins like and/or auxin like substances. Accordingly, the procedure of extraction was intended primarily to look for Indole compounds in the cyanobacterial crude extracts.

Qualitative analysis of IAA and IBA using thin layer chromatography (2D TLC)

To confirm this observation on a chemical basis, the extracts of algae which gave positive effect on growth of rice-wheat seedling, the selected cyanobacterial isolates were analyzed using 2D TLC. The separated spots of algal extracts on the 2D TLC have comparable rate of flow to those of authentic Indole compounds and react positively with the characteristic biochemical reagents of indoles. After 3 hours, the test culture methanol extract was observed which was were very close to standard Indole 3 acetic acid (IAA) and Indole-3-butyric acid (IBA) spots were observed based on R_f value of standard. The relative movement of test sample spot is shown in 2D TLC (Plate 1).

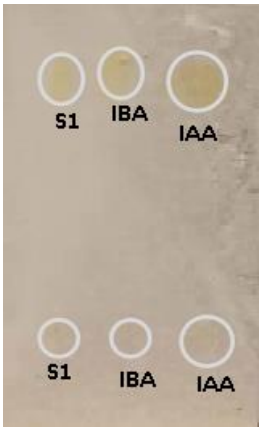

Figure 6: Thin layer chromatography (2D TLC) of cyanobacterial extracted IAA compounds along with standard

The first evidence for the presence of IAA and IBA in the supernatant of cyanobacterial isolates was obtained using 2D TLC where a weak signal co-migrated with the standard IAA and IBA (Plate 1) so, the further confirmation of these results and identification of the endogenously produced indolic substance(s) by cyanobacteria were achieved by HPLC (Figure 5).

IAA confirmation by using High Performance Liquid Chromatography (HPLC)

The HPLC chromatograms of the two microalgae samples under the optimized HPLC conditions were carried out. The use of HPLC equipped with a fluorescence detector showed the presence of two endogenous auxin including IAA, and its homologue, IBA (not shown in plate) in cyanobacterial biomass (Plate 2 and 3).

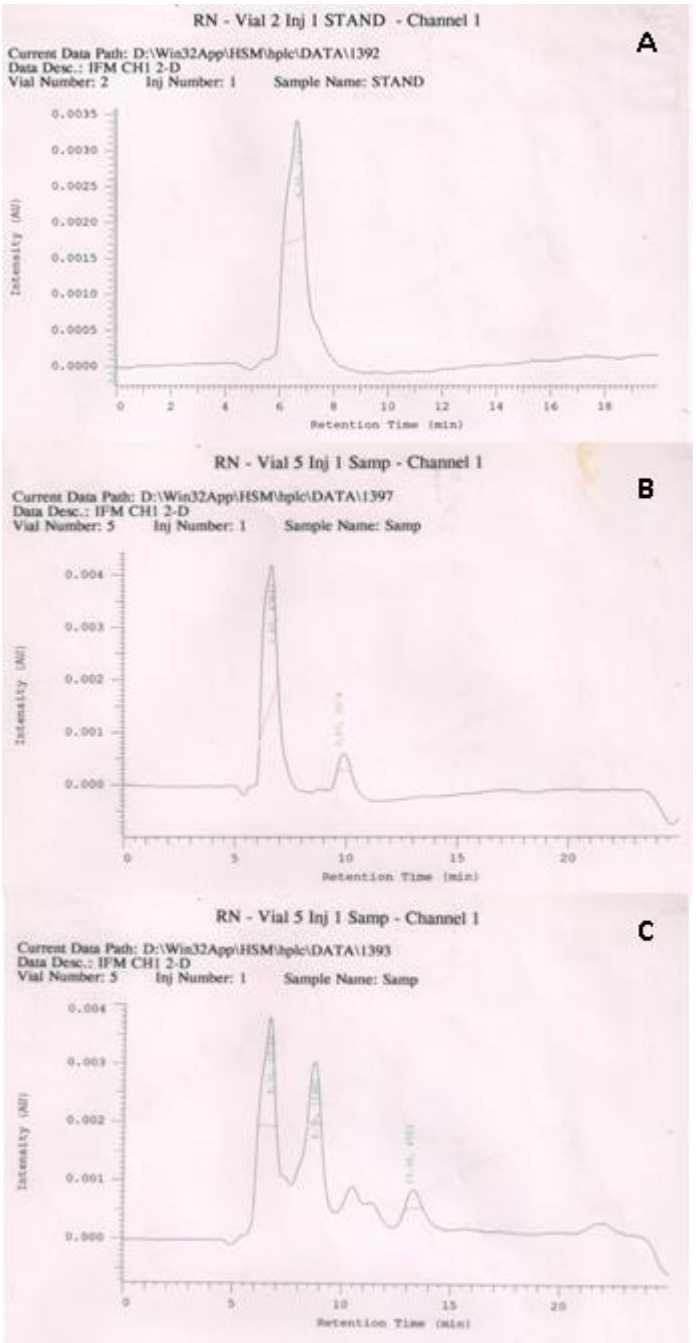

Figure 7: Reverse-phase HPLC profile. A: Authentic indolic compound standards. Retention times shown on X-axis: IAA, 06.69, min. B: Indolic compounds produced by cyanobacterial isolates, retention time B sample is 06.69 min, and for sample C is 06.70min.

The detection of standard indolic compounds showed retention in reverse-phase HPLC profile as shown in HPLC profile A: Authentic indolic compound standards. Retention times shown on X-axis: IAA, 06.69, min. B: Indolic compounds produced by *Chroococcus minutus* (CCC582) cyanobacterial isolate, at retention time 06.69 min, and for reference culture *Nostoc muscurum* (UTEX387) sample the RT was 06.70 min. (Plate 2). These two HPLC identified compounds were further confirmed at third level technique using gas chromatography and gas chromatography mass spectrometry (GC-GCMS).

Present study was focused on 31 different cyanobacterial isolates from rice-wheat organic field were grown and maintained in N_2 free BG-11 medium without tryptophan[11,17] at 27 ($\pm 2^0$)C under light intensity of 52 to 55µmol photon $m^{-2}s^{-1}$ and at 16L:8D (Light:Dark cycle). In present study the IAA production by cyanobacterial isolates was recorded at different concentration of L-tryptophan levels (0, 100, 200, 400 and 500µg/mL) at intermittent light and dark interval. At zero L-tryptophan concentration in 16L:8D exposure the isolate *Microcystis robusta* (CCC568) showed 2.48 (±0.06) IAA µg per ml of culture and in dark *Nostoc* sp. (CCC560) secreted highest 02.67 (±01.300) IAA µg per ml of culture while at 500 µg per ml L-tryptophan concentration in light condition the rice field isolate *Cylindrospermum* sp. (CCC541) showed 15.90±01.26 IAA µg per ml of culture production, where as in dark at same L-tryptophan concentration it was 27.53 (±01.332) IAA µg per ml of culture in *Cylindrospermum* sp. (CCC547), and shows completely inverse results with earlier findings[10,19] but was correlated with our study carried out on cyanobacteria[20,21]. The first scientist who analyzed the culture medium of *Chlorella pyrenoidosa*, *Oscillatoria* sp. and *Anabaena cylindrica*, by extraction with organic solvents and separation by paper chromatography an indolic substance which have a rate of flow near that of IAA and biologically active with Avena coleoptiles test and the same rate of flow was revealed during TLC study with one culture and two standard reference of IAA and IBA[2].

In other study the auxin like compound was extracted from the culture of *Leptolyngbya* strain MMG-1 and identified as Indoe-3-acetic acid (IAA) by thin layer chromatography (2D TLC) and high performance liquid chromatography (HPLC)[22]. On same plat form our study was aimed at the isolation, characterization and identification of IAA and Indoe butyric acid (IBA) compounds from cyanobacterial isolates of 11 different genera. During preliminary identification and characterization, thin layer chromatography (2D TLC) of IAA[15,20] and IBA [16] the cyanobacterial extract was separated on 2D TLC plate coated with 250µm thickness of silica gel. The separated spots of algal extracts on the 2D TLC have comparable rate of flow to those of authentic Indoe compounds (IAA and IBA) and react positively with the characteristic biochemical reagents of Indoles. The further confirmation of endogenously produced Indolic substance (s) was achieved by HPLC and with GC/MS[15]. Analysis of *Anabaena oryzae* exudates by Gas Chromatography Mass Spectrometry (GC-MS) demonstrated the occurrence of gibberellins, n-acetyl-D-glucosamine, linalool, dihydroxyphenyl glycol in addition to niacinamide[23]. The total ion chromatograms of algal extracts showed great similarity and different Indolic substances (IAA and IBA fragments) were separated from all algal extracts at comparable retention times. Released (exogenous) and cellular (endogenous) IAA was extracted from culture supernatant and cells, respectively**[3]**. Cultures were incubated for 3 weeks with 1,000 µg per mL of L-tryptophan supplementation, cells were harvested by centrifugation of 100ml of culture at 10,000 ×g for 20 min at 4^0C and subjected for detection og released IAA and IBA compounds. IAA was extracted from the culture of *A. platensis* strain MMG-9 and in our case it's from *Microcystis robusta* (CCC568) as a analytes where its identity was confirmed by using 2D TLC as well as by HPLC technique[20] and GC/MS technique. During our study the use of HPLC equipped with a fluorescence detector showed the presence of two endogenous auxin including IAA, and its homologue, IBA in cyanobacterial biomass.

Being Plant growth promoting (PGP) organisms cyanobacteria can affect plant growth by different mechanisms *viz*. siderophores production[4] and production of phytohormones such as Indole-3-acetic acid (IAA)[3] which was revealed during seed treatment study where rice seeds treated with 21days old intracellular exudate heterocyst isolate *Cylindrospermum* sp.(CCC541) showed highest seed germination compared to control and reference strain. The existence of growth substance in *Chlorella vulgaris* and its quantity increases when the alga was incubated in the dark and same has been found in our study where the IAA production was higher than light conditions but was exactly opposite with finding of . However the presence of a growth substance similar to IAA was recorded in the excised tissue of *Laminaria agardhii*[24]. A study on cyanobacterial extracts effect on potato (*Solanum tuberosum* L.) tissue confirms that the increase in crop yields can not only be attributed to the nitrogen-fixation potency of cyanobacteria, but may be largely due to the growth regulating

substances endogenously produced by these algae culture[16]. Auxins, especially IBA, are commonly applied to stimulate root initiation and used as plant hormones for the induction and improvement of rooting. Before to this study, increase in lateral root production in the seedlings of several plants with IAA and IBA was reported[25,26 ,27] also showed that phytohormones can promote root elongation and ion uptake in rice seedlings. The results of our study showed that improvement of rooting in the studied rice-wheat seedling plants may be affected by phytohormones such as IAA and IBA.

Free-living *A. platensis* was isolated from a rice paddy[20]. Released (exogenous) and cellular (endogenous) IAA were extracted from culture supernatant and cells, respectively[3]. Cultures were incubated for 3 weeks with 1,000 µg/ml of L-tryptophan supplementation, and for released IAA, cells were harvested by centrifugation of 100ml of culture at 10,000 ×g for 20 min at 4^0C. The filamentous cyanobacterium *Arthrospira platensis* strain MMG-9 was isolated from a rice field. The ability of this strain to synthesize the bioactive compound indole-3-acetic acid (IAA) was demonstrated[20]. IAA was extracted from the culture of *A. platensis* strain MMG-9 and its identity was confirmed by thin-layer chromatography (2D TLC) as well as by high-performance liquid chromatography (HPLC).

Thin-layer chromatography (2D TLC) of IAA was done according (Gravel, 2007), IAA and IBA extracts dissolved in methanol were applied to and developed on silica gel 2D TLC plates using isopropanol/ammonia/water (10/1/1 (v/v/v)) as the mobile phase. The plates were sprayed with a reagent (3% H_2SO_4 in 100 ml of methanol containing 50mg of $FeCl_3$) and heated in an oven at 80°C until color development. IAA appears red under visible light and orange under UV light with Rf values close to 0.60 (pure IAA).

IAA and IBA biosynthesis was measured by using the Salkowski colorimetric technique[28]. The IAA precursor L-tryptophan was required for IAA biosynthesis. Released IAA increased with the increase of the initial concentration of L-tryptophan in the medium and with the incubation time. *A. platensis* strain MMG-9 accumulated more IAA than it released into the medium. But, as compared to seedling response to extracellular exudates the intracellular exudates showed the longest plumule length in rice seedling i.e. with *Nostoc muscorum* (CCC555) showed 12 (±0.64) cm plumule lengths and *Nostoc* sp. (CCC560) showed 11.93 (**±**0.84) cm plumule length followed by treatment with *Nostoc* sp. (CCC546) intracellular exudates showed 12.63 (±1.05) cm plumule length. The bioactivity of the IAA extracted from *A. platensis* strain MMG-9 was demonstrated by assessing the effect of culture supernatant on the root growth of pea (*Pisum sativum*). The bioactivity of the secreted IAA was shown by its effect on the formation of roots by *Pisum sativum*. Seeds were surface sterilized by washing them for 5 min in 0.1% $HgCl_2$, followed by repeated washing with sterile distilled water. Minute changes in the concentration of IAA have an impact on roots of plants. Such responses include the increase of adventitious roots. The length of roots has been used as a bioassay, particularly for IAA (3). After incubation with filtered culture supernatant, seedlings of *Pisum sativum* showed a significant effect on root length as well as on the number of roots[20].

In order to confirm the cyanobacterial secreted IAA the HPLC analysis was done. The use of HPLC equipped with a fluorescence detector showed the presence of two endogenous auxins including IAA, and its homologue, IBA in cyanobacterial biomass. Mass spectrometric data and their comparison with standard compounds confirmed this result[29].

It is of interest to know whether or not the chemical substances acting as hormones in the most highly evolved land plants, such as the angiosperms, are already present in the more primitive plants such as algae. If such chemicals are present in algae, do they function as hormones, or do their hormonal function appear only in land plants. These questions have been answered to some extent concerning the angiosperm hormone IAA[30].

Conclusion

The crude extracts of some non-heterocystous cyanobacterial isolates from organic rice-wheat field have growth promoting effects apart from nitrogen fixing heterocystous cyanobacteria. Auxins are important plant growth regulators which control various aspects of plant growth and development from the cellular to organ and whole plant levels. The algal extracts showing auxin like activities when tested in rice and wheat, showed the secretion growth chemicals which act as endogenous hormones in higher plants, also actually function as growth substances in algae. The increase in crop yields as a result of algal inoculation can not only be attributed to the nitrogen fixing property of cyanobacteria,

but may be largely due to the growth regulating substances endogenously produced by these algae. The cyanobacterial secretion of auxin like substances is independent of light and showed higher IAA production during dark incubation period than light conditions. This suggestion is greatly supported by the fact that non-nitrogen fixing non-heterocystous species like *Microcystis robusta* (CCC568) and *Chroococcus minutus* (CCC582) stimulated the growth of rice and wheat respectively. Results of the present study were in accordance with the previously published data that pointed to the presence of auxin like substances in algae. It could also be concluded that the hormonal function of auxin appears not only in the structurally more complex land plants and the highly differentiated marine algae, but also in structurally simple prokaryotic cyanobacteria. Such algae can be used on a large scale to increase crop plant yields as in case of rice and wheat.

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