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Growth Promoting Substances of Cyanobacteria. II. Detections of Amino Acids, Sugars and Auxins

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The present investigation is based on the analysis of cellular and extra-cellular growth promoting substances liberated by *Nostoc muscorum* and *Hapalosiphon fontinalis* under laboratory conditions. Cyanobacterial extracts stimulated the length of coleoptile and radicle to the extent of > 100% over control (distilled water) and basal medium. Two weeks old cultures produced better stimulation in the length of first leaf, longest root and number of laterals as compared to 4 weeks old cultures.

On analysis of extracellular and cellular substances, the external medium was found to be rich in several amino acids like serine, arginine, glycine, aspartic acid, threonine, glutamic acid, cystine, proline, valine, ornithine, lysine, histidine and isoleucine. In addition to free amino acids, the extra and intracellular polysaccharides were found to be composed of galactose, xylose, fructose and a series of unidentified sugars. The presence of auxin like growth promoting substances was shown in both *Nostoc* and *Hapalosiphon* and their quantities were 3.76 and 4.48 μg/g respectively. Growth promoting substance in *Hapalosiphon* extract was found to be indole-3-acetic acid and the possibility of presence of indole-3-propionic acid or 3-methyl indole also existed. Substances from *Nostoc* could not be partitioned. *Avena* growth test for auxins has indicated the presence of auxins in growth medium of *Nostoc* and *Hapalosiphon* and the quantity was < 0.001 mg/l. The increase in coleoptile length was around five percent.

Key Words: Cyanobacteria, Amino acids, Polysaccharides, Auxins, Rice growth stimulation

Introduction

Nitrogen fixation by cyanobacteria plays a vital role in the buildup and maintenance of soil fertility. Nutrients fixed by the algae are released either through exudation or through microbial decomposition after the cells die. The present communication deals with the analysis of extra- and intracellular substances possibly released by cyanobacteria and involved in the well being of plants.

Materials and Methods

Organism

Nostoc muscorum ARM 221 and *Hapalosiphon fontinalis* ARM 363 were obtained from National Facility for Blue-Green Algal Collection's IARI, New Delhi and were used in the present investigation.

Growth and Maintenance

The cyanobacterial strains were grown in Fogg's (Fogg 1949) medium supplemented with A_5 solution (Arnon 1938) and Fe-EDTA (Jacobson 1951) at 30 $\pm$ 1 °C in an illuminated growth room at 2000 lux. For large quantity of biomass, the alga was grown in 10 litre Aspirator bottles with aeration through oil free compressor.

Rice Growth Tests

Exponentially growing culture was centrifuged at

18,000 rpm for 15 min. The pellet and supernant were collected separately and were treated as Misra and Kaushik (1988). A known (5 ml) volume of concentrated supernatant was used to assess the influence of extracellular substances on rice germlings.

Healthy seeds of paddy variety Pusa-169 were surface sterilized and used for growth tests. The seeds were allowed to germinate in sterile Petri dishes padded with sterile filter paper in dark at 32°C. Germinated/ungerminated seeds, as the case may be, were placed in petridishes containing fixed volume of algal extracts and known volume of standards, and incubated suitably. Medium alone served as controls.

Analysis

Extracellular amino acids: One-month-old culture of cyanobacteria was taken and harvested by centrifugation at 16,000 rpm for 15 min. The supernatant filtrate was concentrated to 10% to its original volume at 35°C in a rotary vacuum evaporator

Amino acids were identified by employing descending chromatography using butanol-acetic acid-water (4 : 1 : 1) as a solvent mixture and ninhydrin as spraying reagent (Kaushik 1987). A chromatogram with developed spots of 10 μg standard amino acids served as reference.

Polysaccharides: The extra- and intracellular polysaccharides were analysed after Moore and Tischer (1965).

Auxins or auxin like substances: Avena straight growth test (Bentley & Housley 1954) was employed for detection of auxins in cyanobacteria. Oat seeds var. kent was used. Graded concentration of IAA (Indole-3-acetic acid) were used as standard solutions. The intracellular auxins were extracted after McDougall and Hillman (1978).

For quantitative estimation, a 2 ml of test sample (standard or unknown) was taken and to it 8 ml of Salkowskis's reagent (conc. H_2SO_4, 0.5 M anhydrous ferric chloride and distilled water in ratio of 8 : 0.15 : 3) was added. The colour was allowed to develop and O.D. was taken at 530 nm. Unknowns were read on a calibration curve plotted with known IAA.

Final identification of IAA was also done with TLC made from Silica gel (Kieselgel G nach Stahl; E. Merck) (Pillay & Mehdi 1988).

Results

Initial Assessment of Response of Paddy to Cyanobacteria

Incubation of healthy seeds of variety Pusa 169 in humidity controlled incubator in dark followed by monitoring the germination percentage at 2 and 3 days interval suggested that soaking of the seeds in cyanobacterial cell suspension culture filtrate, growth medium alone or distilled water did not affect the germination percentage significantly (results not included). Therefore, the influence of cellular and extracellular substances of cyanobacteria was examined on 3 days germinated seeds. The length of coleoptile was 0.008 or 0.038 cm and of radicle 0.153 or 0.215 cm in presence of distilled water or basal medium respectively. These length were significantly improved in presence of *N. muscorum* and *H. fontinalis*. In presence of *Nostoc* extracts the length of coleoptile and radicle were 1.792 and 4.261 cm, while due to *Hapalosiphon* it was 2.20 and 4.961 cm respectively. Therefore, the extracts from the cyanobacteria were examined in greater detail.

Influence of Age and Period of Incubation of Cyanobacteria on Rice Germlings

The substances from 2 weeks old cultures were added to 4 days old rice germlings and observed after 3 days of incubation showed that a significant differences were seen only in the length of first leaf and length of longest root. The cellular and extracellular extracts from *Nostoc* were superior over *Hapalosiphon* (table 1).

The extracts from 4 weeks of old cyanobacterial culture provided better differences as compared to 2 weeks old culture. The differences began to appear also in the length of coleoptile and radicle of rice germlings (results not included). The length of first leaf was 3.35 or 3.05 cm due to cellular extracts of *Nostoc* or *Hapalosiphon* respectively in 4 weeks old alga as compared to 2 weeks old alga which stimulated 3.20 or 2.45 cm respectively only. However, due to extracellular substances a reduced stimulation was seen (table 1).

On extending the period of incubation for 5 days instead of 3 days with other things constant, the differences in stimulation due to algal extracts were still more prominent. The length of the longest root varied from 3.7-4.3 cm due to cellular than 1.7 cm due to extracellular substances from *Nostoc* and *Hapalosiphon*.

On the contrary, number of laterals showed better differentiation with extracellular substances. The length of first leaf also showed similar response.

With keeping the age of rice germling and period of incubation constant and taking the extracts from 4 weeks old culture, almost identical trend was observed. The length of longest root and number of leaves were again better influenced by cellular extracts than extracellular (table 1).

With increase in the period of incubation second and third leaves began to appear and thus the period of incubation was not extended to any further. The extracts were characterized for various biologically potent substances.

Amino acids: It is clear from the results given in table 2, that a variety of amino acids were liberated by cyanobacteria during the growth phase. The presence of threonine, glutamic acid, proline and valine were well established in external growth medium of *N. muscorum.* In addition to these, glycine or aspartic acid and serine or arginine were also liberated. The R_f value of these amino acids was overlapping between arginine, serine, glycine and aspartic acid. The free amino acids liberated by *Hapalosiphon* were little different from that of *Nostoc,* as the presence of lysine, cystine, and isoleucine was detected in addition to arginine, serine, aspartic acid and glycine. There existed a possibility of ornithine or cystine also in extracellular extracts. The presence of histidine was observed only in *Hapalosiphon.* No attempts were made to hydrolyse the extracts for additional identification of amino acids.

Sugars: With the techniques available (paper and TLC) no free sugars could be detected in the extra cellular extracts. In the extracellular polysaccharides of *N. muscorum* after suitable hydrolysis only the presence of galactose and xylose was observed. Four unidentified sugars were also observed with the R_f values of 0.017, 0.057, 0.104 and 0.324. The sugars in the extracellular polysaccharides of *H. fontinalis* were better identified as compared to *Nostoc* and the presence of galactose, fructose and xylose along with an unidentified sugar of R_f value 0.022 was detected (table 3).

Growth hormones: Initial test for the presence of auxin like substances in cyanobacteria was done with Salkowski's reagent. From the initial tests it seems that *H. fontinalis* is richer than *N. muscorum* in terms of the presence of growth hormones. Quantitatively *Hapalosiphon* contained 4.48 μg of auxin like compounds per g of fresh biomass as compared to *Nostoc* which had only 3.76 μg/g fresh alga (table 4).

In an attempt to purify the auxin-like substances, partially purified extracts were further partioned employing TLC. On repeatedly run chromoplates, extracts from *Nostoc* failed to give any positive tests (colour spots) with Urk's reagent.

Table 1 *Influence of cellular and extracellular substances of cyanobacteria (2 and 4 weeks old) on rice germlings (4 and 5 days) after 3 and 5 days of incubation (Average of 10 germlings)*

Incubation (days)	*2 weeks old age*											
	Cellular						*extracellular*					
	Hapalosiphon			*Nostoc*			*Hapalosiphon*			*Nostoc*		
	1	2	3	1	2	3	1	2	3	1	2	3
3	2.45	1.90	5.5	3.20	1.55	5.5	2.45	1.10	9.0	3.05	1.95	10.5
5	2.8	3.7	6	3.2	4.3	8	2.5	1.7	11	3.6	1.7	10
	4 weeks old age											
3	3.05	1.45	6	3.35	1.60	8	2.0	0.55	2.0	2.0	1.0	3.0
5	3.5	3.4	6	3.1	2.1	9	3.1	2.0	7	2.1	2.1	8

1, length of longest leaf (cm), 2, length of longest root (cm), 3, number of laterals.

Table 2. *Extracellular amino acids of* ***N. muscorum and H. fontinalis.***

N. muscorum	*H. fontinalis*
Serine/Arginine/Glycine	Ornithine/Cystine/?
Glycine/Aspartic acid	Lysine
Threonine/?	Cystine
Glutamic acid	Arginine/Histidine
Proline/?	Serine/Aspartic acid/Glycine
Valine	Isoleucine

Table 3 *Composition of cyanobacterial polysaccharides*

N. muscorum		*H. fontinalis*	
Cellular	*Extracellular*	*Cellular*	*Extracellular*
Galactose	Galactose Xylose	Galactose Xylose	Galactose Xylose Fructose
	Unknown with R_f		
0.014	0.017	0.015	0.022
0.057	0.057	0.078	
0.097	0.104	0.370	
0.377	0.324		

Table 4 *Colorimetric estimation of auxin/auxin like substances in cyanobacteria (average of 3 replications)*

	Transmission % (530 nm)	*IAA (μg/ml)*
	100	0.0
	97.6	0.1
	88.3	0.2
	79.3	0.5
	72.2	1.0
	65.0	2.0
	53.4	4.0
N. muscorum	38.4	3.76*
H. fontinalis	47.4	4.48*

*Based on fresh biomass

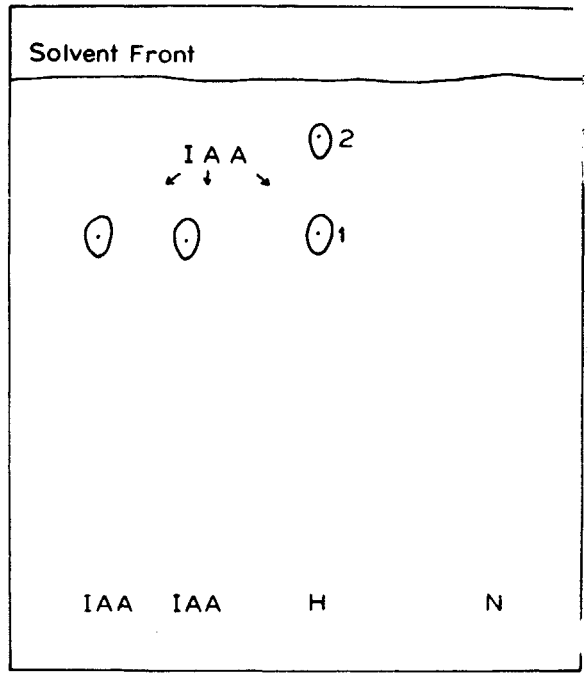

Figure 1 Separation of intracellular auxins by TLC using chloroform: acetic acid (95 : 5 v/v) Solvent system (IAA, Indole-3-acetic acid; H, *Hapalosiphon* extract; N, *Nostoc* extract; Spot 1, IAA; Spot 2, Indole-3-propionic acid/3-Methyl indole).

However, extracts from *Hapalosiphon* gave two spots of blue and violet colour. On comparing the R_f of standard (IAA) and unknowns, it was observed that R_f value of unknown (spot 1) was very close to the R_f of indole-3-acetic acid, and the second (spot 2) was either indole-3-propionic acid or 3-Methyl-indole, based on the standard R_f values available (figure 1).

Avena Growth Test

A confirmatory evidence was further provided by Avena growth test. The results are given in table 5. It was observed that with the increase in the concentration of IAA from 0 to 10 mg/l, the percentage increase in the coleoptile length was perceptible upto a concentration of 0.1 mg/l. The increase in the length was from 5.81 to 13.95 %. Beyond the 1 mg/l concentration, there was a drop in the increase of coleoptile length. The increase in the length of coleoptile due to *N. muscorum* and *H. fontinalis* over the control was 5.23 and 4.65 percent respectively and was very close to the increase due to 0.001 mg/l IAA.

Table 5 *Avena growth test for bioassay of extracellular cyanobacterial auxins vis-a-vis known auxin (IAA) (Average of 10 replications)*

IAA (mg/1)	*Coleoptile length (mm)*	*Increase over control (%)*
0	8.60	—
0.001	9.10	5.81
0.01	9.15	6.39
0.1	9.8	13.95
1.0	9.65	12.21
10.0	9.25	7.56
N muscorum	9.05	5.23
H fontinalis	9.00	4.65

Discussion

Evidences on production of hormonal substances by cyanobacteria has come primarily from treatment of rice seedlings with algal cultures or their extracts (Gupta & Lata 1964). A significant increase in the length of coleoptile and radicle was observed due to whole cell (cellular + extracellular substances) extracts of *H. fontinalis* followed by *N. muscorum*. A similar influence on the growth of roots and shoots has been earlier shown (Gupta & Shukla 1969). From the four sets of experiments i.e., the influence of extracts from 2 and 4 weeks old cultures on 4 days rice germlings after 3 or 5 days of incubation, an interesting fact emerged that the age of the alga plays a greater role than the period of incubation of rice germlings. From the results, it was evident that extracellular substances of 2 weeks old cyanobacteria have more pronounced influence on growth as compared to 4 weeks old cultures. This may be because that at late log or stationary phase, alga may be producing certain growth inhibiting substances (Gorham 1960, Kato et al. 1962) or due to reduced synthesis of growth promoting substances.

The probable nature of substances responsible for stimulation of growth of rice has been linked to that of gibberellin (Gupta & Shukla 1969), however, a variety of other substances are being synthesized and released during the growth phase or on the death of alga. The fixed nitrogen is released in the form of free state (Venkataraman & Saxena 1963, Mohan & Mukherji, 1978), and in the form of peptides (Fogg 1952, Whitton 1965). Qualitatively almost all types of amino acids, as a source of nitrogenous substances, are released extracellularly by cyanobacteria. Amino acids recorded in the culture filtrates of *N. muscorum* and *H. fontinalis* during the present investigation were almost identical to those as reported earlier. The possible liberation of arginine, histidine, isoleucine, lysine, threonine and valine is of significance since these amino acids are a part of essential amino acids required for human nutrition (table 2). Stimulation of root growth of rice has also been shown due to tyrosine, phenllalanine, cystine and glycine (Venkataraman & Neelakantan 1967).

Apart from enriching the environment with nitrogenous substances, cyanobacteria also contribute carbonaceous material. Cells of blue-green and green algae form mucilaginous capsules, presumed to be of polysaccharide nature. In addition, it has been observed that algae liberate soluble organic material in the medium. Qualitatively, the polysaccharides were composed of galactose, arabinose, glucose, xylose, fructose, rhamnose and several other sugars and lipids (Moore, & Tischer 1965, Weise et al. 1970). The polysaccharide from *N. muscorum* and *H. fontinalis* were also composed of galactose, fructose, xylose and several unidentified sugars (table 3). The role of such organic molecules in nature may be various *viz.* (i) improvement in the soil structure, (ii) may act as chelating agents for heavy metals, (iii) may stimulate growth of heterotrophic bacteria which in turn produce indirect effect on plant growth, and (iv) may be assimilated directly by plant roots as preformed organic substrates.

The growth of rice seedlings treated with algal filtrates from *Aulosira fertilissima* were to show the pattern with the seedlings treated with gibberellic acid (Singh & Trehan 1973). Initial indications for the presence of auxin like substances in *Nostoc* and *Hapalosiphon* were obtained with Salkowski's reagent (table 4). Similar positive indication for the presence of auxin like substances in *Anabaenopsis raciborskii* was obtained by Mohan Mukerji (1978). The extract from *Nostoc* failed to give any separation, and thus compound could not be identified, whereas in *Hapalosiphon*, the presence of indole-3-acetic acid (IAA) was confirmed. An additional spot was also present whose R_f value was very close to indole-3-propionic acid or 3-methyl indole. The absence of identifiable growth promoting substances

in *Nostoc* may be due to the presence of inhibitors along with growth promoting substances which were not subjected to vigorous purification procedures as earlier reported (Kato et al. 1962).

By employing the *Avena* growth test bioassay under present investigation, an increase in the length of coleoptile was measured. The increase due to *Nostoc* culture filtrate corresponded close to 0.001 mg/l pure IAA, while in *Hapalosiphon,* the concentration seems to be < 0.001 mg/l. Therefore, the present studies have indicated the liberation of amino acids, sugars (polysaccharides) and growth promoting hormonal substance by *Nostoc* and *Hapalosiphon.* The absence of any coloured spot in extracts of *Nostoc* does not rule out the presence of growth promoting substances and this belief was substantiated by *Avena* growth test bioassay

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References

Arnon D I 1938 Microelements in culture solution experiments with higher plants; *Amer. J. Bot.* **25** 322-325

Bentley J A and Housley S 1954 Bioassay of plant growth hormones; *Physiol. Plant* **7** 405-419

Fogg G E 1949 Growth and heterocyst production in *Anabaena cylindrica* II. In relation to carbon and nitrogen metabolism; *Ann. Bot.* **13** 241-259

—— 1952 The production of extracellular nitrogenous substances by a bluegreen alga; *Proc. R. Soc. Lond.* **B139** 372-397

Gorham P R 1960 Toxic waterblooms of blue-green algae; *Can. Vet. J.* **1** 235

Gupta A B and Lata K J 1964 Effect of algal growth hormones on the germination of paddy seeds; *Hydrobiologia* **24** 430-434

—— and Shukla A C 1969 Effect of algal extracts of *Phormidium* species on growth and development of rice seedlings; *Hydrobiologia* **34** 77-84

Jacobson L 1951 Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamino tetra-acetate; *Pl. Physiol.* **26** 411-413

Kato J Purves W K and Phinney B O 1962 Gibberellin-like substances in plants; *Nature* **196** 687

Kaushik B D 1987 *Laboratory Methods for Blue-green Algae* (New Delhi: Associated Publ. Co.) p. 184

McDougall J and Hillman J R 1978 Analysis of indole-3-acetic acid using GCMS technique; in *Isolation of Plant growth Substances* pp 1-25 ed. J R Hillman (Cambridge: Cambridge University Press)

Misra S and Kaushik B D 1988 Growth promoting substances of cyanobacteria. I. Vitamins and their influence on rice plants; *Proc. Indian natl. Sci. Acad.* B (In press).

Mohan M and Mukerji K G 1978 Some biologically active extracellular products of blue-green algae; *Phykos* **18** 73-82

Moore B G and Tischer R G 1965 Biosynthesis of extracellular polysaccharides by blue-green alga *Anacystis flos-aquae; Can. J. Microbiol.* **11** 877-885

Pillay D T N and Mehdi R 1968 Separation of simple indole derivatives by thin layer chromatography *J. Chromatog.* **32** 592

Singh V P and Trehan T 1973 Effect of extracellular products of *Aulosira fertilissima* on the growth of rice seedlings; *Plant Soil* **38** 457-464

Venkataraman G S and Neelakantan S 1967 Effect of cellular constituents of the nitrogen fixing blue-green alga *Cylindrospermum muscicola* on the root growth of rice plants; *J. Gen. Appl. Microbiol.* **13** 53-61

—— and Saxena H K 1963 Studies on nitrogen fixation by blue green algae. IV. Liberation of free amino acids in the medium; *Indian J. agric. Sci.* **33** 22-24

Wada H Panichsakpatana S Kimura M and Takai Y 1978 Nitrogen fixation in paddy soils. I. Factors affecting nitrogen fixation; *Soil Sci. Plant Nutr.* **24** 357-365

Weise G Drewes G Jann B and Jann K 1970 Identification and analysis of a lipopolysaccharide in cell wall of blue-green algae *Anacystis nidulans; Arch. Mikrobiol.* **71** 89-98

Whitton B A 1965 Extracellular products of blue-green algae; *J. gen. Microbiol.* **40** 1-11