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Nitrogen metabolism, artificial association study in two cyanobacterial isolates and assessment of their potential as biofertilizer

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Two strains of cyanobacteria, viz. *Nostoc* ANTH and *Mastigocladus* sp, were isolated from local separate temperature zones of Meghalaya, India. Both the strains showed preference for different temperatures for optimum growth [45°C for *Mastigocladus* sp.(thermophile) and 25°C for *Nostoc* ANTH (mesophile)]. The addition of nitrogen sources in the growth media (nitrate, ammonia and glutamine) supported their better growth but repressed heterocyst development and nitrogenase activity. Nitrate and nitrite uptake rates, NR and NiR activities increased by NO_3^- and decreased by NH_4^+ in *Nostoc* ANTH. However, such effects were only partial in *Mastigocladus* sp. The presence of fixed nitrogen sources in the media led to decreased GS activity and repressed methylammonium uptake in both the strains. Glutamine uptake was substrate inducible, energy-dependent and required *de novo* protein synthesis. Artificial association studies revealed successful establishment of association of rice roots with both cyanobacteria, including prolonged association of *Mastigocladus* sp. at high temperature (~45°C). Little modifications in growth temperature and growth media led to profuse akinete differentiation in target cyanobacteria. The replacement of normal cells by akinetes as field inoculants might have profound biotechnological implications in future biofertilizer programme.

Keywords: cyanobacteria, N_2-fixation, mesophile, thermophile, biofertilizer
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Introduction

Cyanobacteria, an extremely diverse group of Gram-negative prokaryotes, show diversity in physiology, morphology, developmental characteristics and habitats[1-4]. In nature, they are free-living as well as symbiotic[3,5]. Cyanobacteria, as diazotrophs, have a long history of usage as biofertilizer in agriculture[6-8] and are known to enrich the nitrogen content of the rice fields[9]. Species of *Nostoc, Anabaena, Aulosira, Tolypothrix, Cylindrospermum, Gloeotrichia, Gloeocapsa, Anabaenopsis, Camptylonema, Scytonema* and *Westiellopsis* are widespread in Indian soils and rice fields, contributing immensely to soil fertility. The current biofertilizer programme using free-living cyanobacteria poses many problems, including low survival rate of inoculum; adaptability of cyanobacteria inoculated in the fields with regards to competition with the preexisting natural populations; and incompatibility with chemical fertilizers and low nitrogen release. Also, most cyanobacteria have optimum N_2-fixing ability in a temperature range of 20-30°C. Therefore, the mesophilic cyanobacteria may not be the ideal source of biofertilizer in tropical rice fields where daytime temperature can soar anywhere 30-45°C, and this adversely affects their metabolic processes. Under these conditions, thermophilic cyanobacteria seem to be the better option as biofertilizer in mixed consortia. Information about thermophilic cyanobacteria that grow at such high temperatures is scanty and so far has not been looked into as the potential biofertilizer. Keeping this in mind, the mesophilic *Nostoc* ANTH and thermophilic *Mastigocladus* sp., both isolated from the state of Meghalaya, were compared with respect to selected metabolic processes with an aim to enumerate their use as potential biofertilizer in rice fields of different temperature zones.

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Materials and Methods

Strains and Culture Conditions

Nostoc ANTH was isolated from the undersurface of the gametophytic thalli of the bryophyte

Anthoceros, collected from the North Eastern Hill University campus, Shillong and purified under axenic conditions. Axenic cultures were grown in batches and maintained in BG-11_0 (N_2-medium)[10] at 25°C with a photon fluence rate of 50 µmol m^{-2} s^{-1}. Whenever needed, the N_2-medium was supplemented with 5 m*M* KNO_3 (NO_3-medium) or 2 m*M* NH_4Cl (NH_4-medium) or 1 m*M* L-glutamine (glutamine-medium).

Mastigocladus sp. was isolated from the hot spring of Jakrem, Meghalaya state, India and grown in axenic batch cultures in D-medium[11] under continuous light with a photon fluence rate of 50 µmol m^{-2} s^{-1} at 45°C in BOD incubator and also at 25°C in the culture room. Whenever needed, cultures were also grown in N_2-medium (D-medium without source of combined nitrogen), NH_4-medium (N_2-medium supplemented with 2 m*M* NH_4Cl) or in N_2-medium supplemented with 1 m*M* L-glutamine. All media for *Nostoc* ANTH and *Mastigocladus* sp. were buffered with equimolar concentration of HEPES, and *p*H was adjusted to 7.5 before autoclaving.

Growth, Heterocyst Frequency and Nitrogenase Activity

Growth was measured as increase in concentration of *Chl a*[12]. Heterocyst frequency in both the cyanobacteria was calculated as percentage of total vegetative cells after 96 h of incubation in different nitrogen media. Acetylene reduction assay was used to measure nitrogenase activity[13].

Nitrate and Nitrite Uptake Assay

The N_2-, NO_3- and NH_4-grown cultures of *Nostoc* ANTH and *Mastigocladus* sp. were harvested by centrifugation during the exponential growth phase (after 4 d growth), washed and resuspended in Tricine-$NaHCO_3$ buffer (25 m*M*, *p*H 8.1). Uptake experiments were initiated by the addition of $NaNO_3$ (100 µ*M*) or KNO_2 (100 µ*M*) to the cell suspension. Uptake of NO_3^- and NO_2^- was measured by the rates of their depletion from the medium. The choice of 100 µ*M* external concentration for both the combined N sources was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942[14,15]. Samples were withdrawn after 3 h of incubation, subjected to rapid centrifugation at 5000 g and the cell-free supernatants analyzed for residual NO_3^- or NO_2^-. Nitrate and nitrite concentrations were measured by the method of Cawse[16] and Snell and Snell[17], respectively.

Nitrate Reductase, Nitrite Reductase and Glutamine Synthetase (Transferase) Activity

Ferredoxin-dependent nitrate reductase (NR) and nitrite reductase (NiR) activities were measured using dithionite reduced methyl viologen as reductant[18,19]. Glutamine synthetase (transferase; GS) activity was measured as described by Sampio *et al*[20]. However, in *Mastigocladus* sp., incubation for the enzyme assays was performed at higher temperature (45°C) for cultures grown at 45°C. In case of *Nostoc* ANTH, cultures were incubated at 30°C. Protein was measured according to Lowry *et al*[21].

Ammonium and Amino acid Transport Assay

Ammonium transport assay for *Nostoc* ANTH and *Mastigocladus* sp. was done using the radioactive [^{14}C]-methylammonium, an analogue of ammonium (sp. activity 370 KBq µmol^{-1})[22]. Glutamine uptake was measured using [^{14}C]-glutamine (sp. activity 256 KBq µmol^{-1}) for both the cyanobacterial strains. NO_3-grown cultures were harvested during the exponential growth phase, washed in N_2-medium and incubated in N_2-medium or N_2-medium supplemented with 1m*M* glutamine at 25°C for *Nostoc* ANTH and at 45°C for *Mastigocladus* sp. After 48 h, cells were harvested, washed and resuspended in 10 m*M* HEPES-NaOH buffer (*p*H 7.0) to a final concentration of 10 µg mL^{-1} *Chl a* for both the strains and equilibrated for 1 h under their respective growth conditions. Methylammonium or glutamine uptake experiments were started by adding [^{14}C]-methylammonium or [^{14}C]-glutamine to a final concentration of 50 µ*M* for both the cyanobacteria. Whenever needed, dichlorophenyldimethylurea (DCMU, 10 µ*M*) or carbonyl cyanide chlorophenyl hydrazine (CCCP, 25 µ*M*) was added to the cell suspension 30 min prior to the addition of labelled glutamine. At different time intervals, 400 µL sample was taken out quickly and cells separated by centrifugation through silicon oil DC 550/dinonylphthalate (40/60, v/v) into perchloric acid/water (15/85, v/v)[22] and [^{14}C] in the perchloric acid fraction measured using liquid scintillation counter (Beckman, Model 1801). The non-specific binding of [^{14}C]-methylammonium and glutamine was determined by measuring their incorporation in the toluene treated cells[22].

Cultures Conditions for Akinetes Differentiation

Nostoc ANTH cultures were induced to sporulate in BG-11_0 medium lacking $MgSO_4$ and replaced by equimolar $MgCl_2$. *Mastigocladus* sp., on the other

hand was found to sporulate spontaneously in N_2-medium at lower temperatures. Akinete frequency was calculated as the percentage of total cells in Olympus BX-51 light microscope fitted with a JVC digital video camera.

Co-cultivation of Rice and Cyanobacteria

Rice seedlings (10-d-old) grown on perlite were transferred to 15 mL culture tubes after washing with distilled water. D-N_2-medium and BG-11_0 medium (10 mL) was poured independently into these tubes and seedlings carefully placed with the roots dipped in the medium. *Mastigocladus* sp. and *Nostoc* ANTH were then inoculated in these test tubes in their respective growth media. Tubes with *Mastigocladus* sp. were kept in the BOD incubator at 45°C and with *Nostoc* ANTH in the growth chamber at 25°C.

Results

Growth, Heterocyst Frequency and Nitrogenase Activity

Both *Nostoc* ANTH and *Mastigocladus* sp. were grown initially in a temperature range of 20 to 60°C. Growth was measured as increase in *Chl a* content on 4th d. *Mastigocladus* sp. grew best at 45°C (2.3 µg mL^{-1} *Chl a* in D-N_2-media). However, *Nostoc* ANTH cells died at temperatures exceeding 35°C and showed optimal growth at 25°C (1.2 µg mL^{-1} *Chl a* in N_2-media). Growth was consistently higher in NO_3, NH_4 or glutamine supplemented media. Heterocyst frequency and nitrogenase activity were not detected in combined nitrogen supplemented media. The growth, heterocyst frequency and nitrogenase activity of *Mastigocladus* sp. were ~1.5-fold higher at 45°C than at 25°C.

Nitrate and Nitrite Uptake Activities

Both *Mastigocladus* sp. and *Nostoc* ANTH showed NO_3^- and NO_2^- uptake activities. The rate of NO_3^- uptake by *Mastigocladus* sp. grown in N_2-medium was 27.04 nmol min^{-1} mg^{-1} *Chl a* compared to 32.9 nmol min^{-1} mg^{-1} *Chl a* in NO_3-medium or 8.8 nmol min^{-1} mg^{-1} *Chl a* in NH_4-medium (Table 1). Further, NO_3^- uptake by *Nostoc* ANTH was 2.8 nmol min^{-1} mg^{-1} *Chl a* in N_2-medium, 3.6 nmol min^{-1} mg^{-1} *Chl a* in NO_3-medium and 0.4 nmol min^{-1} mg^{-1} *Chl a* in NH_4-medium. Hence, there was 21% and 28% increase in NO_3^- uptake by *Mastigocladus* sp. and *Nostoc* ANTH, respectively in the NO_3-medium as compared to N_2-medium. Interestingly, NO_3^- uptake rate of *Mastigocladus* sp. was ~10-fold higher compared to *Nostoc* ANTH under similar conditions of N_2- and NO_3-medium. However, the presence of NH_4^+ in the growth medium led to inhibition of NO_3^- uptake, which was more severe in case of *Nostoc* (88%) compared to *Mastigocladus* sp. (73%) than their respective N_2-grown cells.

On the other hand, NO_2^- uptake by *Mastigocladus* sp. and *Nostoc* ANTH was increased by 40 and 20%, respectively in NO_3-medium as compared to N_2-medium (Table 1). However, severe inhibition in NO_2^- uptake by *Nostoc* ANTH (99%) than in *Mastigocladus* sp. (39%) was noticed in the presence of NH_4^+.

NR, NiR and GS Activity

In NO_3-medium, NR activity showed a drastic increase (133%) in *Nostoc* ANTH (25°C) against a moderate increase (17%) in *Mastigocladus* sp. (45°C) in comparison to its activity in N_2-medium (Table 2). However, the NR activity declined by 88% in *Nostoc* ANTH and 46% in *Mastigocladus* sp. in the presence of NH_4^+ in the medium.

In N_2-medium, NiR activity in *Nostoc* ANTH was much higher (540 nmol NO_2^- consumed min^{-1} mg^{-1} protein) as against the enzyme activity in *Mastigocladus* sp. (163 nmol min^{-1} mg^{-1} protein). NiR activity showed a 38% rise in *Nostoc* ANTH in NO_3-medium in comparison to activity in N_2-medium, which by contrast was only 8% in *Mastigocladus* sp. However, the presence of NH_4^+ in the medium led to 57% inhibition of NiR activity in *Nostoc* ANTH, but the inhibition was only 6% in *Mastigocladus* sp. (Table 2).

A comparative study of GS activity revealed that *Mastigocladus* sp. had much higher enzyme activity (2464 nmol γ-glutamyl hydroxamate min^{-1} mg^{-1}

Table 1—Nitrate and nitrite uptake by *Nostoc* ANTH and *Mastigocladus* sp. pre-grown in media containing different nitrogen sources

Nitrogen sources in growth medium	Nitrate uptake (µmol nitrate taken up min^{-1} mg^{-1} *Chl a*)		Nitrite uptake (µmol nitrite taken up min^{-1} mg^{-1} *Chl a*)	
	Nostoc ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.
N_2	2.8 ± 0.2	27.04 ± 0.4	24.02 ± 1.1	21.74 ± 1.0
NO_3	3.6 ± 0.1	32.92 ± 1.6	29.26 ± 0.9	30.41 ± 1.5
NH_4	0.4 ± 0.1	8.81 ± 0.4	0.2 ± 0.1	17.29 ± 0.8

N_2 = No combined nitrogen; NO_3 = + 10 m*M* $NaNO_3$; NH_4 = + 2 m*M* NH_4Cl

Table 2—Effect of different nitrogen sources on nitrate reductase (NR), nitrite reductase (NiR), and glutamine synthetase (transferase) (GS) activities of *Nostoc* ANTH and *Mastigocladus* sp.

Nitrogen sources in growth medium	NR activity (nmol NO_2^- formed min^{-1} mg^{-1} protein)		NiR activity (nmol NO_2^- consumed min^{-1} mg^{-1} protein)		GS activity (nmol γ-glutamyl hydroxamate formed min^{-1} mg^{-1} protein)	
	Nostoc ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.
N_2	1.8 ± 0.1	9.16 ± 0.4	540 ± 27	162.53 ± 8	610 ± 7	2464 ± 123
NO_3	4.2 ± 0.1	10.07 ± 0.5	745 ± 37	175.03 ± 8	598 ± 3	1689 ± 84
NH_4	0.2 ± 0.1	4.87 ± 0.2	230 ± 12	152.65 ± 7	376 ± 6	1466 ± 73

NR = Nitrate reductase; NiR = Nitrite reductase; GS = Glutamine synthetase
N_2 = No combined nitrogen, NO_3 = + 10 m*M* $NaNO_3$, NH_4 = + 2 m*M* NH_4Cl.

protein) compared to *Nostoc* ANTH (610 nmol γ-glutamyl hydroxamate min^{-1} mg^{-1} protein) grown in N_2-medium. However, GS activity was repressed by 31% in *Mastigocladus* sp. as against 2% in *Nostoc* ANTH when NO_3^- was added to the growth medium. The addition of NH_4^+ to the medium also showed similar inhibitory effect on the GS activity in *Mastigocladus* sp. (40%) and in *Nostoc* ANTH (38%) (Table 2).

Methylammonium and Glutamine Uptake

The status of ammonium uptake in *Nostoc* ANTH and *Mastigocladus* sp. was studied using [^{14}C]-methylammonium. Both the organisms showed a biphasic pattern of methylammonium uptake marked by an initial rapid phase lasting for 60 sec, followed by a slower second phase that remained linear during the next 10 min of the experimental period. Methylammonium uptake activity in *Nostoc* ANTH during the first and the second phase was 55 and 7.3 nmol mg^{-1} min^{-1}*Chl a*, respectively. The uptake rates for the same compound in *Mastigocladus* sp. cells were 42 and 15.77 nmol mg^{-1} min^{-1} *Chl a* during the first and second phase, respectively. The addition of NO_3^- and NH_4^+ in the growth-medium led to severe repression of the methylammonium uptake in both the cyanobacteria. Overall, the data showed that nitrogen starvation increases ammonium transport activity in both the strains.

Studies on the uptake of glutamine in *Nostoc* ANTH and *Mastigocladus* sp. also showed a biphasic nature where an initial rapid phase represented intracellular accumulation, followed by a slower second phase representing assimilation. The glutamine uptake rates were higher in glutamine-grown cells than in N_2-grown cells. Such an increase in the rates was significantly inhibited by the addition of chloramphenicol, an inhibitor of protein synthesis (Fig. 1). Also, DCMU and CCCP inhibited the glutamine uptake in both the strains (Table 3).

Table 3—The effect of DCMU and CCCP on [^{14}C]-glutamine uptake by *Nostoc* ANTH and *Mastigocladus* sp.

Growth-medium	[^{14}C]-Glutamine uptake (nmol [^{14}C]-glutamine taken up mg^{-1} min^{-1} *Chl a*)	
	Nostoc ANTH	*Mastigocladus* sp.
Control	44.0 ± 2.2	131.71 ± 6.5
Control + DCMU (10 μ*M*)	21.8 ± 1.0	51.21 ± 2.0
Control + CCCP (25 μ*M*)	3.8 ± 0.1	21.87 ± 1.5

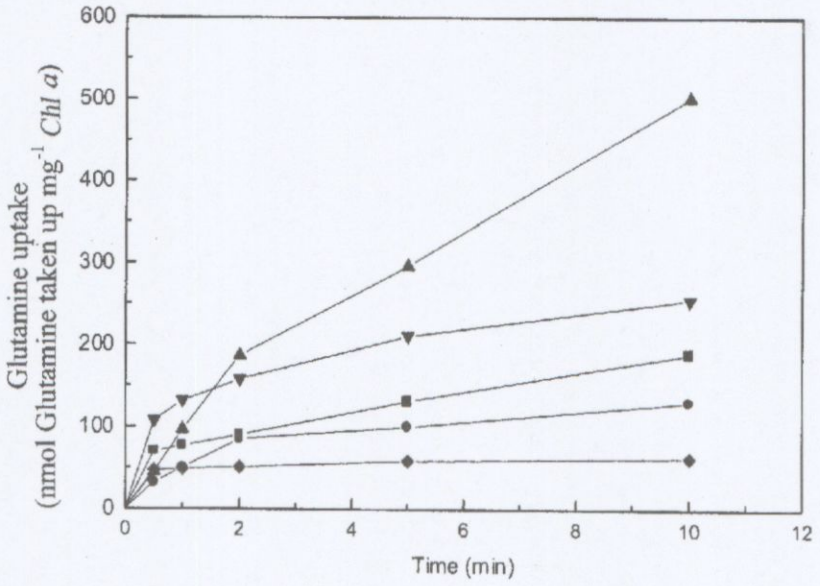

Fig 1—[^{14}C]-Glutamine uptake in *Nostoc* ANTH grown in N_2- (25°C, ●) and glutamine-medium (25°C, ▲); and *Mastigocladus* sp. grown in N_2- (45°C, ■), glutamine-medium (45°C, ▼) and glutamine-medium+chloramphenicol (♦). (NO_3-grown exponential cells were washed and transferred to N_2- and glutamine-medium (1m*M* glutamine) and incubated (48 h) at 25°C for *Nostoc* ANTH and at 45°C for *Mastigocladus* sp. Cells were then harvested, washed and resuspended in HEPES buffer and used for glutamine uptake as described in Materials and Methods. The values are means of two independent experiments, each with two replicates).

Akinete Differentiation

The interest in akinete was for its potential as inoculum to be used to populate rice fields with competent cyanobacterial strains. Akinete differentiation in *Nostoc* ANTH started only when the

N_2-medium was used to grow the cells lacked either sulphate or phosphate. the former limitation leading to quicker and higher extent of akinete differentiation (within 3-4 d as against 8-9 d). In contrast, akinetes in *Mastigocladus* sp. differentiated with in 10 d simply when grown in D-N_2-medium at lower temperature (25°C; Table 4). By 40th d, the akinetes percentage of *Nostoc* ANTH in sulfate limited N_2-medium and *Mastigocladus* sp. in normal D-N_2-medium were 98 and 80%, respectively at 25°C. Some degree of akinete differentiation (~20%) was also observed by 40th d in *Mastigocladus* sp. grown at 45°C probably due to exhaustion of media nutrients.

Table 4—The time course of akinete differentiation in *Nostoc* ANTH (25°C) in sulfate limiting N_2-medium and *Mastigocladus* sp. in normal D-N_2 medium (25°C and 45°C)

Time (d)	Akinete frequency (%)		
	Nostoc ANTH	*Mastigocladus* sp.	
	25°C	25°C	45°C
0	0.0	0.0	0.0
5	48 ± 1.0	0.0	0.0
10	59 ± 1.0	12.0 ± 0.6	0.0
20	78 ± 1.0	30.0 ±1.5	0.0
30	92 ± 1.0	52.0 ± 2.6	0.0
40	98 ± 1.0	80.0 ± 4.0	20 ± 1.0

Artificial Association of Rice Plants with Cynobacteria

To test whether these two cyanobacteria can be used as biofertilizer, *Mastigocladus* sp. and *Nostoc* ANTH were incubated with rice seedlings. There was considerable association of both these cyanobacteria at room temperature (25°C). However, when incubated at 45°C, *Nostoc* ANTH died after 24 h. Interestingly, *Mastigocladus* sp. showed significant association with rice roots even at 45°C for prolonged period. N_2-fixation activity was measured after 7 d of incubation. It was higher in associated cells of both the cyanobacteria. This activity was 16.28 and 12 nmol C_2H_4 produced μg^{-1} h^{-1} *Chl a*, respectively in associated and free-living cells of *Nostoc* ANTH at 25°C. The corresponding values for *Mastigocladus* sp. at 45°C same were 6.27 and 4.62 nmol C_2H_4 produced μg^{-1} h^{-1} *Chl a*.

Discussion

Morphologically, *Nostoc* ANTH and *Mastigocladus* sp. are different cyanobacterial genera; *Nostoc* ANTH without branching and *Mastigocladus* sp. with true branching. Growth experiments conducted beyond 35°C led to cell death in *Nostoc* ANTH. However, there was increase in growth, heterocyst frequency and nitrogenase activity in *Mastigocladus* sp. at 45°C and beyond, indicating its thermophilic nature. Both the cyanobacteria were able to utilize, NO_3^-, NO_2^-, NH_4^+ and glutamine as sole N source for growth but these were inhibitory to heterocyst differentiation and nitrogenase activity. These observations are similar to earlier reports in other cyanobacteria[23-28]. In general, the appearance of the *Mastigocladus* sp. was more yellowish than of the *Nostoc* ANTH. This could be due to the fact that the media composition used for growth of *Mastigocladus* sp. lacked in one or more nutrient(s) that otherwise is available to the organism in the hot spring. A comparison of heterocyst frequency and nitrogenase activity in the two cyanobacterial genera at their respective growth temperatures showed that *Nostoc* ANTH had higher nitrogenase activity even though heterocyst frequency was higher in *Mastigocladus* sp. probably due to more efficient N_2-fixing and/or C-fixing machinery in the former.

Further, investigations into nitrogen metabolism aspects of the two isolates established that nitrate and nitrite uptake rates were inducible by NO_3^- and repressible by NH_4^+. However, nitrate uptake rates of *Mastigocladus* sp. were much higher (~10-fold) than of *Nostoc* ANTH. As is the case with other cyanobacteria[26], the severity of inhibition by NH_4^+ was more pronounced in *Nostoc* ANTH. However, in contrast to a complete repression of nitrate and nitrite uptake by NH_4^+ in other cyanobacteria[26,29], it was only partial in *Mastigocladus* sp.

Again, NR activity followed the trend of other cyanobacteria for being NH_4^+ repressible-depressible[30-33], and NO_3^- and NH_4^+ had visible inductive and repressive effects, respectively on the NiR activity of *Nostoc* ANTH. In contrast, these effects were negligible (<10%) in case of *Mastigocladus* sp. Even though this report on NiR activity in *Nostoc* ANTH is similar to other cyanobacteria[25], it is in complete contrast with regard to *Mastigocladus* sp. Such observations may be of importance, as *Mastigocladus* sp. under field conditions may be able to adapt in a much better way against the nitrate-based chemical fertilizers load in the fields.

Energy-dependent cellular accumulation of glutamine, as evident by inhibitory effects of DCMU or CCCP on glutamine uptake as well as the comparable effects on GS activity and

methylammonium uptake in the presence of fixed nitrogen sources in both the cyanobacteria, are consistent with the earlier reports on other cyanobacteria[24-27,34,35]. Thus, the comparative study of various aspects of nitrogen metabolism in *Nostoc* ANTH and *Mastigocladus* sp. showed that the two isolates from this region have similarities in the process apart from minor differences possibly due to their origin from different temperature and nutrient regimes.

Survival, nitrogen fixation and adaptability of various metabolic processes to different temperatures prompted further investigations into both the cyanobacteria for their ability to associate and also fix atmospheric N in the associated state with selected crop plants. As rice is the prime cash crop of the region and cyanobacteria are known to thrive well in the waterlogged rice fields, artificial association studies were carried out using these two cyanobacterial species with the rice seedlings. While *Nostoc* ANTH showed significant association with rice roots up to a temperature range of ~35°C and not beyond, *Mastigocladus* sp. showed association even at temperatures beyond ~35°C for prolonged period. The extent of nitrogen fixation was also almost one and half-fold higher in the associated cyanobacteria compared to the free-living cultures. This fact tempted us to presume that in future biofertilizer research, these two cyanobacterial isolates can be exploited as the potential biofertilizer candidates in varying temperature regimes.

Therefore, *Nostoc* ANTH with its high nitrogenase activity could be the effective biofertilizer for rice fields of temperate regions, while *Mastigocladus* sp. could be for tropical rice fields where day temperatures can go as high as 40-45°C. However, more studies are needed to ascertain the N-transfer potential of both these strains to rice plants as biofertilizer.

In the current biofertilizer programme, distribution of cyanobacterial inoculum is still a problem, as generally the inoculum comprises fresh cyanobacterium cells where most of these perish in the packing, storage and distribution. During the current study, one interesting observation was the ability and ease with which both these cyanobacteria differentiated high percentage of akinetes. As akienetes can withstand adverse environmental conditions, they can also probably adapt better than the fresh cyanobacterial inocula mainly owing to the stress during the packaging period. Therefore, akinetes can be viewed as the potential candidate to populate rice fields with N_2-fixing cyanobacteria. This could also tackle the low survival rate of inoculum in the rice fields. Also, once they are in the field conditions over a considerable period of time as akinetes, they probably would be better adapted to compete with the natural populations of other microbes in the fields. As reported earlier[36,37], *Nostoc* ANTH can fix nitrogen in associated state in NO_3-medium in dark and therefore, it can be used along with nitrate based chemical fertilizers in the fields. Currently, we are looking into various aspects of N_2-fixation in *Mastigocladus* sp. associated with rice roots in different N-media to ascertain the flexibility of N_2-fixing ability in the cyanobacterium and whether like *Nostoc* ANTH, *Mastigocladus* sp. can also be used as biofertilizer in rice fields in the presence of nitrate based chemical fertilizers. Further, we are looking into developing technology to induce quick sporulation in these cyanobacteria in an attempt to study the stability of the akinetes when stored over a longer period of time, their efficient germination into viable cyanobacterial cells and the retention of N_2-fixing ability of cultures. If the long-term storage is viable for akinetes, it will certainly open up the possibility of easy transportation and distribution of desired cyanobacterial inoculum as akinetes from the place of origin to the areas of application. Further, methods need to be developed to engineer direct delivery of effective strains endowed with enhanced N releasing capacity to the target crop plants for a fruitful N_2-fixing association.

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