Evaluating the potential of *N. calcicola* and its bicarbonate resistant mutant as bioameleorating agents for ‘usar’ soil

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Received: 22 December 2007 / Accepted: 30 January 2008

Abstract The potential of *Nostoc calcicola* and its bicarbonate resistant mutant as bioameleorating agent was investigated, using laboratory simulation experiments, in terms of their growth potential, glutamine synthetase (GS) activity, heterocyst frequency and effect on pH of soil. *Nostoc calcicola*, exhibited a tendency to lower the pH of ‘usar’ soil significantly and showed better growth and pigment content at 20% soil extract as compared to basal medium. The bicarbonate resistant mutant (HCO_3^- -^R) exhibited a better ability to grow at higher percentage of soil extract (60%), besides bringing about a more significant change in soil pH as compared to wild type. The heterocyst frequency was much higher in the mutant strain, which was not significantly affected by growth in various concentrations of soil extract. The mutant strain holds promise as a potential bioameliorant for ‘usar’ soil after further evaluation of its reclamative properties at field level.

Keywords Bicarbonate resistant mutant · Bioameliorant · Cyanobacteria · *Nostoc calcicola* · Usar soil

Introduction

Cyanobacteria or blue green algae (BGA) are well established as major contributors to soil fertility of rice fields besides being widely distributed in diverse environments [1]. As pioneers of inhospitable environments, they possess the ability to colonize bare areas of rocks and soil, and play a critical role in the re-vegetation of such habitats [1, 2].

Usar soil is saline and alkaline soil, characterized by impermeability, extreme hardness and occasional presence of undesirable salts, which makes the soil unproductive. Reclamation of such soils requires the removal of exchangeable sodium or its replacement with calcium. This is normally done through the application of suitable chemical corrections, such as gypsum, followed by leaching which is a rather expensive method and does not completely remove the soluble salts and exchangeable sodium in soil. Another approach for the reclamation of ‘usar’ soil is through microbial activity. Singh [2] reported that in alkaline ‘usar’ soil of northern India, where other plants fail to grow, cyanobacteria (BGA) formed a thick stratum on the soil surface during the rainy season and retreating monsoon, and could be used for reclaiming such soil. Apte and Thomas [3] observed a 26–38% decline in salinity after repeated cultivation of *Anabaena torulosa* in such soil. Improvement of soil properties by reduction in pH, electrical conductivity (EC), exchangeable sodium and hydraulic conductivity has been reported [4]. Blue green algae are also known to improve soil aggregation [5, 6].

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N. calcicola is an 'usar' land isolate, which has the capacity to tolerate high concentration of bicarbonate (up to 250 mM) in the medium [7]. The mutant strain of the cyanobacterium was able to tolerate four folds higher concentration NaHCO_3 as compared to the wild type [7]. Significant variations in morphological [8] and biochemical responses [9] were observed between wild type and mutant strains, reflecting a defective carbon concentrating mechanism (CCM) in the mutant strain, which requires bicarbonate supplementation in the medium for optimal growth. The current investigation was undertaken to study the role of the bicarbonate resistant mutant in reclamation of 'usar' soil using laboratory simulation studies.

Materials and methods

Organism and growth conditions

Nostoc calcicola Breb, an 'usar' land isolate, was cultivated and maintained in combined nitrogen-free Allen and Arnon medium with A_5 trace elements and Fe-ethylene diamine tetra acetic acid (EDTA) as iron source [10]. The cultures were incubated at $25 \pm 1^\circ\text{C}$ with photon flux density of $25 \mu\text{Em}^{-2}\text{s}^{-1}$. The pH of the medium was maintained initially by using 10 mM HEPES buffer. The bicarbonate resistant mutant of the cyanobacterium was isolated by chemical mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [7].

Protein estimation

Protein content was measured by Lowry's method as described by Herbert et al. [11].

Dry weight

Cyanobacterial samples were dried at 80°C in an oven until constant weight was obtained.

Estimation of photosynthetic pigments

Spectrophotometric evaluation of chlorophyll *a* and carotenoids in cyanobacterial cells was carried out by following the method of Myers and Kratz [12].

2, 6-dichlorophenol indophenol (DCPIP) reduction

DCPIP reduction was measured as a index of photosynthetic activity by method of Halt and French [13] using spheroplasts [14] prepared from cyanobacterial cells.

Carbohydrate content

Anthrone reagent was used to determine simple sugars and their polymers by the method described by Herbert et al. [11].

Glutamine synthetase (transferase) activity

The transferase activity of glutamine synthetase (GS) was measured colorimetrically following the method of Shapiro and Stadtman [15].

Preparation of soil extract and monitoring cyanobacterial growth

Soil samples were collected from 'usar' land in the month of June when no apparent growth of algae or cyanobacteria was noticed on soil surface. Soil was powdered, dried and sieved. Deionised water (500 ml) was added to 500 g of soil in a flask (1 L). The mixture was steamed for 30 min in an autoclave and then cooled overnight. The process was repeated twice and finally the solution was filtered through Whatman filter paper (No. 42), and soil extract obtained was sterilized and supplemented with Allen and Arnon medium at different concentrations. The cultures of wild type and mutant strain of *N. calcicola* were inoculated at an inoculum density of $20 \mu\text{g protein ml}^{-1}$ and the cultures were incubated under optimal growth conditions for 10 days.

Heterocyst frequency (HF)

Heterocyst frequency was calculated using the formula

$$\text{HF} = \frac{n(\text{H})}{n(\text{V}+\text{H})} \times 100$$

where V and H represent the number of vegetative cells and heterocysts, respectively and n denotes number.

Statistical analysis

Statistical analysis (two-way ANOVA) was done according to Snedecor and Cochran [16] and at least three independent experiments were conducted.

Results

Effect of NaHCO_3 concentration on wild type and mutant strain of *N. calcicola*

The wild type strain showed better growth (in terms of protein content), photosynthetic activity, carbohydrate content at ambient atmosphere as compared to the mutant strain

Table 1 Effect of bicarbonate concentration on growth and physiology of wild type and HCO_3^- -R mutant strains of *N. calcicola*

NaHCO ₃ concentration (mM)	Protein content (μg ml ⁻¹)		*DCPIP reduction μ mol μg ⁻¹ Chlorophyll <i>a</i> min ⁻¹		Carbohydrate content (μg mg ⁻¹ dry wt)		GS-transferase activity (n mol γ-glutamyl hydroxymate mg protein min ⁻¹)	
	Wild type	HCO ₃ ^{-R} mutant	Wild type	HCO ₃ ^{-R} mutant	Wild type	HCO ₃ ^{-R} mutant	Wild type	HCO ₃ ^{-R} mutant
Control	72 ± 5.8	64 ± 2.4	0.90 ± 0.04	0.88 ± 0.03	108 ± 9.8	95 ± 7.2	445 ± 35.2	410 ± 27.7
50	125 ± 11.0	107 ± 7.0	1.20 ± 0.03	0.92 ± 0.03	123 ± 14.0	118 ± 8.0	815 ± 37.2	610 ± 15.1
75	198 ± 10.0	128 ± 6.9	1.44 ± 0.06	1.23 ± 0.05	154 ± 20.0	138 ± 6.9	885 ± 18.5	680 ± 31.4
100	157 ± 13.1	159 ± 13.1	1.35 ± 0.05	1.40 ± 0.09	180 ± 13.1	154 ± 13.1	830 ± 63.6	730 ± 42.0
250	54 ± 02.0	215 ± 11.6	0.6 ± 0.05	1.56 ± 0.09	80 ± 8.0	194 ± 21.6	435 ± 26.9	790 ± 24.2
500	-	176 ± 05.8	-	1.15 ± 0.05	-	141 ± 5.8	-	750 ± 24.2
1000	-	54 ± 05.2	-	0.62 ± 0.02	-	60 ± 5.2	-	370 ± 22.5

Protein content, carbohydrate content and GS-transferase activity were estimated after 5 days of growth in respective bicarbonate concentrations.

*Cultures were grown in respective bicarbonate concentrations for 5 days, washed, centrifuged and pellets were used for preparation of spheroplasts.

(Table 1). However with increasing concentration of bicarbonate, the response of the mutant strain became better than that of the wild type. At 250 mM NaHCO₃, the growth and photosynthetic response (DCPIP reduction) of the mutant strain was respectively 4.2 and 2.6 fold higher than its wild type. Further increase in the concentration of NaHCO₃ to 500 mM, resulted in death of the wild type strain. Increase in bicarbonate concentration caused an enhancement in GS activity of both strains. The enzyme activity of the mutant strain was comparatively lower than the wild type at their respective optimal NaHCO₃ concentration.

Effect of cyanobacterial growth on pH of 'usar' soil

The effect of cyanobacterial growth (wild type and mutant strain) was evaluated using different concentrations of soil extract and medium (after 10 days of inoculation). The wild type strain modified the pH of the control medium from 7.8 to 9.2 after 10 days of growth whereas the bicarbonate-resistant mutant increased the pH to 8.7 (Table 2). Cyanobacterial growth also brought about a significant change in the pH of 'usar' land soil + medium in different combinations. The pH of soil extract alone also changed considerably from 10.23 to 9.8 and to 8.9 after the growth of wild type and mutant strains, respectively.

Effect of 'usar' land soil extract on growth and pigment content of cyanobacteria

The cyanobacterium *N. calcicola*, after ten days of growth in Allen and Arnon medium showed a protein content of 252 μg ml⁻¹, which served as control for the other treatments (Fig. 1a). Combinations of 'usar' land soil extract and

Table 2 Effect of cyanobacterial (*Nostoc calcicola*, wild type and HCO₃^{-R} mutant) growth on pH of 'usar' soil extract supplemented medium

Concentration of soil extract(%, v/v)	Initial pH	Final pH*	
		Wild type	HCO ₃ ^{-R} mutant
Control	7.8	9.28	8.65
5	9.85	9.42	8.76
10	10.02	9.43	8.79
20	10.11	9.48	8.82
40	10.15	9.60	8.89
60	10.16	9.72	8.92
80	10.17	9.80	8.92
100	10.23	9.86	8.96

*pH was recorded after 10 days of growth in respective soil extract.

medium, supported the growth of the cyanobacterium. Maximum growth of the cyanobacterium was recorded in 20% soil extract and the protein content and dry weight (Fig. 1b) of the strain was 2 and 2.6 folds higher than the control, respectively. Increasing concentration of soil extract further to a level of 40 and 60% gradually decreased the growth of the cyanobacterium, however, protein content/dry weight was still higher than the control. The HCO₃^{-R} mutant also exhibited maximum growth in 20% soil extract, thereafter, there was a gradual decline in the growth. However, the growth of the mutant strain was invariably higher than wild type at all levels of the soil extract supplementation, except control condition. Soil extract alone (100%) suppressed the growth of both the strains but growth of the mutant strain was 3 folds higher than the wild type. The two-way analysis of variance

Fig. 1. Effect of usar land soil extract on growth [(a) protein content, (b) dry weight] of wild type and HCO_3^- mutant strains of *Nostoc calcicola*. Vertical lines on each bar represent $\pm$ SD.

showed that the growth (protein content/dry weight) differed significantly in wild type and mutant strain at different levels of soil extract supplementation. A significant interaction was found between concentration of soil extract in the medium and protein content ($F_{7,1} = 27.506$; $p < 0.001$) or dry weight ($F_{7,1} = 19.9$; $p < 0.001$) of the two strains.

The pigment content of wild type and mutant strain also followed a similar trend, i.e maximum pigment content was recorded at 20% soil extract, thereafter it showed a gradual decline (Fig. 2a, b). But unlike protein content/dry weight, pigment content of mutant strain was lower than the wild type at lower concentrations (0–40%). Higher soil extract concentrations supported higher pigment content in mutant strain as compared to the wild type. The statistical analysis (two-way ANOVA) revealed that pigment content differed significantly in wild type and mutant strain grown in different levels of soil extract. [Chlorophyll *a* ($F_{7,1} = 55.38$; $p < 0.001$); carotenoids ($F_{7,1} = 5.58$; $p < 0.001$)]

Effect of soil extract on heterocyst frequency

No significant change in heterocyst frequency was observed at different concentration of soil extract supplementations. Heterocyst frequency of wild type ranged from 5.7 to 6.0, while that of mutant strain ranged from 8.2 to 9.0 (Table 3).

Discussion

Soil salinity is a widespread problem especially in India where around 6.7 million ha is affected by coastal salinity and inland alkaline and saline soil ('usar') covering an area of about 6.6 million ha [17]. The reclamation of these soils is of immense practical importance to bring back millions of acres of such unproductive lands once again under the plough. A number of BGA (presently

Fig. 2. Effect of usar land soil extract on pigment [(a) Chlorophyll *a* and (b) carotenoids content of wild type and HCO_3^- mutant strains of *Nostoc calcicola*. Vertical line on each bar represent $\pm$ SD.

known as cyanobacteria) grow luxuriantly in alkaline/saline soil [18], and their abundant growth produces striking and beneficial changes in such soils. Cyanobacteria are also well known to accumulate inorganic carbon 1000 folds higher than in external medium [19]. *Nostoc calcicola*, being the native strain of the 'usar' land soil, has an inherent capability to tolerate high concentrations of bicarbonate in the medium and its bicarbonate resistant mutant (HCO_3^- -R) can tolerate four folds higher NaHCO_3 as compared to the wild type [7]. A significant effect of bicarbonate incorporation was found on the cell volumes of wild type and mutant strain, and cell volume of mutant strain was invariably found higher than wild type, which might be due to higher intracellular influx of bicarbonate ions [8].

A comparative analysis of growth characteristics of wild type and mutant strain at ambient atmosphere revealed that the mutant required higher concentration of bicarbonate in the medium for growth [7, 9], which reflects a defective CCM in the mutant strain which functions optimally at higher level of bicarbonate. This enhances the suitability of mutant strain in removal of salt from the soil crust. Further the GS-transferase activity in the mutant strain was found to be lower than wild type at ambient atmosphere and also at their respective optimal NaHCO_3 concentration (75 and 250 mM for wild type and mutant strain, respectively). The rate of photosynthesis, measured as DCPIP reduction showed an enhancement with increase in the concentration of NaHCO_3 in both wild type and mutant strain. This is expected, as increase in NaHCO_3 would result in higher CO_2 concentration

Table 3 Effect of usar land soil extract on heterocyst frequency* of *Nostoc calcicola* and its bicarbonate resistant mutant in soil extract supplemented medium

Concentration of soil extract (% v/v)	Heterocyst frequency	
	Wild type	HCO ₃ ^{-R} mutant
Control	6.0	9.0
5	6.0	8.9
10	5.8	8.4
20	5.9	8.2
40	5.7	8.6
60	6.0	8.8
80	5.8	8.8
100	5.8	8.4

*Heterocyst frequency was determined after 6 days

(the ultimate electron acceptor), this in turn would further lead to increased photolysis of water/DCPIP reduction. However, the photosynthetic activity of the mutant strain was higher than that of the wild type at their respective optimal NaHCO₃ concentration indicating that although the mutant strain has sufficient carbon skeletons for incorporation of nitrogen it is unable to utilize them. It can be hypothesized that the mutant strain might be defective in GS and may possess the property of liberating ammonia.

pH is one of the important factors determining the cyanobacterial biodiversity and abundance in any environment [20, 21]. In culture media, the optimal pH for cyanobacterial growth ranges from 7.5–10, the lower limit being 6.5–7.0 [22]. Under natural conditions, BGA grow optimally in environments that are neutral to alkaline [23] and sometimes the growth of cyanobacteria in rice fields is limited by low pH [2]. The poor growth of N₂ fixing BGA in acidic soil is probably due to the inability to compete with Chlorophycean algae, which are favored by acidic conditions. The high pH in 'usar' soil supports the growth of cyanobacterial populations, which in turn brings about a decline in the pH of usar land soil. In the present investigation, both wild type and mutant strains caused a significant drop in pH of 'usar' soil extract following their growth, which is in consonance with the observation of Singh [18]. The presence of high pH, Na⁺ ions and soluble CO₂ in these soils provide a favorable environment for inorganic carbon accumulation for these cyanobacteria. The role of Na⁺ in bicarbonate uptake is well known [19, 24, 25], and the increase in growth of both the strains in different concentration of soil extract may be attributed to increasing photosynthetic and N₂ fixing activities due to higher concentration of inorganic carbon

and Na⁺. Also, pH of the medium showed an enhancement with increasing concentration of soil extract, which may in turn enhance N₂ fixation. *N. calcicola* has been demonstrated to grow and fix nitrogen maximally at a pH of 9.5 [26] and N₂ fixing capacity of this organism continues till pH 10.5 [27]. At higher soil concentrations (40–100%), the growth performance of the mutant strain was much better than the wild type indicating that the mutant strain is better suited to grow in usar soil. Also, it has a better tolerance capacity to higher salt concentration than wild type. The mutant strain is known to synthesize a stress protein in the presence of high salt concentration [9], which might be of added advantage for the mutant strain in order to survive and grow in usar land soil. It can be surmised that the property of N₂ fixation, in a way, forces these cyanobacteria to fix more CO₂, thereby leading to efficient functioning of nitrogenase.

Diazotrophic cyanobacteria play a key role in maintaining the nitrogen budget as well as fixing CO₂ from the atmosphere. The mutant (defective in CCM) evaluated in this study may be exploited for reducing the salt concentration of the usar soil, and the addition of organic matter by their growth can lead to improvement of soil permeability, aeration and other physical properties. The significance of such mutants in the reclamation of usar soil is tremendous, as they not only buffer the pH so as to make them conducive for the growth of plants and microorganisms, but also play an important role as CO₂ sinks.

Acknowledgments

The authors are thankful to Head, Department of Botany, Banaras Hindu University for infrastructure facility and the Council of Scientific and Industrial Research (CSIR), for providing financial assistance to carry out research activities. The facilities required provided by the Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi is also gratefully acknowledged.

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