IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
February 2018 Vol.:8, Issue:4

Development of a Salt-Resistant Mutant of *Anabaena variabilis* for Soil Reclamation

IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

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Submission: 21 January 2018
Accepted: 29 January 2018
Published: 28 February 2018

Keywords: Salt stress, cyanobacteria, *Anabaena variabilis*, mutation, salt-resistant strains

ABSTRACT

A salt-tolerant mutant of *Anabaena variabilis* was prepared and isolated by treatment with N-methyl-N'-nitro-N" -nitroguanidine (NTG) and maintained in the BG11 liquid medium. The salt tolerant strain showed morphological variations, higher growth, and higher frequency of nitrogen-fixing cells, heterocyst's as compared to its wild counterpart at the NaCl concentration of 300 mM in the medium. The NaCl mutant can be more useful in paddy fields where the soil has been deteriorated due to high salt concentrations.

INTRODUCTION:

Soil salinity is an important agricultural problem and high salt concentration is a practical deterrent to plant growth in saline habitats. The photoautotrophic nitrogen-fixing cyanobacteria, in general, are believed to increase nitrogen fixation in paddy fields all over the world. These cyanobacteria exhibit considerable tolerance to salt or osmotic stress and reclamation of saline/sodic soils using these organisms have been attempted all over the world (Bohnert and Jensen, 1996).

Over 7 million hectares of land area is estimated to be affected by salts in India, posing serious ecological and agronomical problems (Apte et al., 1990). These areas include Indo-Gangetic plains, black cotton soils (prevailing in Madhya Pradesh) and the entire Indian coast. Such lands are increasingly becoming inhospitable to crop production, although chemical amendments like the application of gypsum, followed by leaching with good quality irrigation water are being used at some places for reclamation.

As global temperatures rise, rainfall patterns shift, and ecosystem nutrient dynamics change, cyanobacteria are expanding their ranges and gaining dominance in phytoplankton communities in aquatic systems worldwide. Cyanobacteria provide distinct advantages over higher plants and plant-based systems as experimental materials. In paddy fields all over the world, cyanobacteria form the substantial fraction of the blooms in submerged soils, and provide a good amount of nitrogen to the rice plants, helping in improving the crop yield considerably. Majority of these cyanobacteria grow in freshwaters of paddy fields and are intolerant to salt stress. In saline environments, this factor results in low crop yield as well as crop quality.

The basal salt acclimation strategy of cyanobacteria includes two principal reactions, the active export of ions and the accumulation of compatible solutes. Cyanobacterial salt acclimation has been characterized in much detail using selected model cyanobacteria, but their salt sensing and regulatory mechanisms are less well understood (Pade and Hagemann, 2014).

The present study is the first step to isolate a salt tolerant mutant for the paddy fields of non-coastal areas, with increased ability to fix nitrogen under higher salt stress. The study is

highly significant for the reclamation of the soil, deteriorated with high salt concentrations over the years due to saline water as well as overuse of fertilizers and pesticides.

MATERIALS AND METHODS

Isolation of wild-type strain of *Anabaena variabilis*

The wild-type strain of *Anabaena variabilis* was isolated from a paddy field near Jabalpur (MP). The cyanobacterial mat that consisted more than 90% of *A. variabilis* was brought to the laboratory. A small portion of the mat was teased with fine needles and vortexed for 5 seconds to disintegrate the filaments. The suspension was diluted serially and streaked on BG11 agar medium. The plates were kept in a culture room at 25 ±2 °C temp, 50 µmol $m^{-2}s^{-1}$ light on the surface and a 16:8 hours the day: night cycle. Once the filaments started growing, a single filament was picked up using a needle and subcultured. The process was repeated till a unialgal culture was obtained. The cyanobacteria were identified as *Anabaena variabilis* based on microscopic characters (Desikachary, 1960). The unialgal cultures were maintained in BG11 liquid media for further experiments.

Mutagenesis and isolation of NaCl mutant strain

Exponentially growing cells of *A. variabilis* were harvested by centrifugation and resuspended in 10 mM HEPES/NaOH buffer, pH=7.0. The filaments were sonicated in a sonication bath (Soniprep, MSE, India), until filaments were reduced to an average length of two cells (as seen under the microscope), followed by two washes in 10 mM HEPES/NaOH buffer, pH=7.0. N-methyl-N'-nitro-N"nitroguanidine (NTG) in a final concentration of 250 µg ml^{-1} was added to the suspension (Gour et al., 1997). The suspension was incubated at 30°C for 4 h under standard light conditions with constant stirring. The mutagenic treatment was terminated by washing the cells thrice with BG11 medium and finally suspend them in the same media.

For isolation of the mutagenic strain of *A. variabilis*, the mutagenized cells were grown photoautotrophically for several generations on to BG11 medium solidified with 1% agar and containing 300 mM NaCl, a concentration which completely arrested the growth of wild-type strain. Colonies appearing on to the plate were taken up and grown for many generations in

BG11 medium with 10 mM HEPES/NaOH, pH 7.0 and 300 mM NaCl. This strain was designated as *A. variabilis* $NaCl^r$

Measurement of growth

The cyanobacterial growth was measured by the increase in chlorophyll *a* content and was expressed in terms of specific growth rate (μh^{-1}). The specific growth rate constant (μ) corresponds to 2/td, where td is the doubling time.

$$\mu = \frac{2.303 \times (\log N_2 - \log N_1)}{(T_2 - T_1)}$$

Where: N_1= initial chlorophyll content

N_2= Final Chlorophyll content

T_1= Initial time

T_2= Final Time

Nitrogen-fixing ability

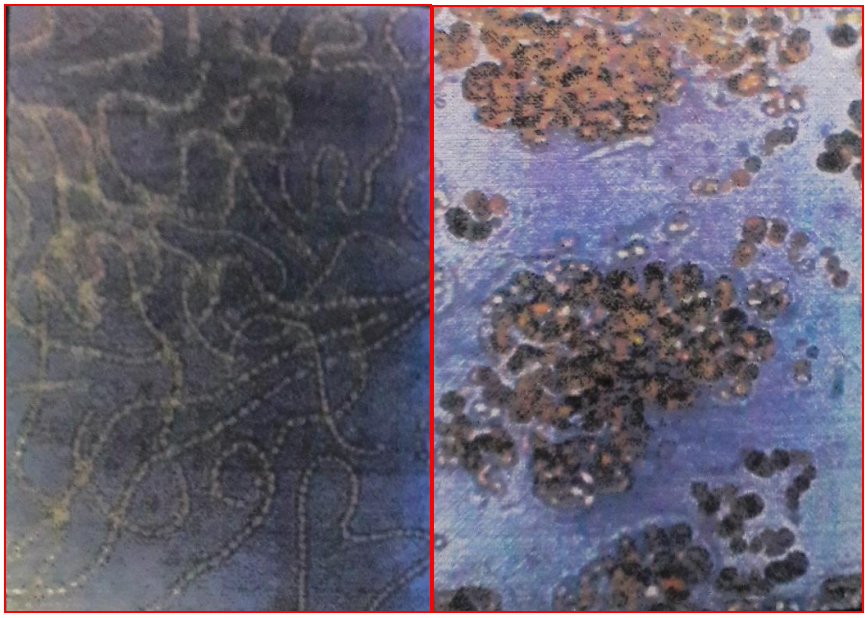
The ability of wild type and mutant *A. variables* to fix nitrogen was assessed by heterocyst frequency microscopically using ten replicates and expressed as the number of heterocysts per 100 vegetative cells (Chouhan et al., 1999).

RESULTS AND DISCUSSION

The $NaCl^r$ mutant strain of diazotrophic cyanobacteria *A. variabilis* (a rice field isolate) was obtained by NTG mutagenesis and screened for growth and nitrogen-fixing ability in the medium containing 300 mM NaCl, the concentration at which growth of its wild-type in liquid medium was completely arrested within 2 weeks.

The $NaCl^r$ mutant strain, in contrast to its parent strain, showed morphological variation by forming multicellular structure without branching (Fig 1), which may probably be due to abnormal division in all planes unlike to its wild-type strain (Chouhan et al., 1999). Similar

altered cell divisions leading to abnormal pattern formation have also been reported in cyanobacteria following NTG treatment (Angadi and Datta, 1992).



A **B**

Fig 1: A: wild-type strain of *Anabaena variabilis* showing heterocysts and vegetative cells, B: NaCl^r mutant strain showing multicellular structure without branching.

Table 1 presents the data on growth and heterocyst frequency of wild-type and NaCl^r mutant strain of *Anabaena variabilis* in nitrogen-rich BG11 medium, in the presence and absence of NaCl (300 mM). It is evident from results that wild-type grew reasonably well by utilizing N_2 as the nitrogen source, produced heterocysts (6%) in absence of NaCl. The NaCl^r mutant strain could also grow well at the expense of N_2 with a growth rate similar to its wild-type counterpart but produced more heterocysts (8%). NaCl at 300 mM concentration severely affected growth and heterocyst differentiation in wild type, whereas the same treatment stimulated these activities significantly in NaCl^r mutant. The most likely reason for NaCl induced higher growth and heterocyst frequency in NaCl^r mutant strain appears to be due to enhanced availability of cellular nitrogen reserve for fresh protein synthesis required for growth as well as for production of new heterocysts. Since Na^+ uptake was found to be energy dependent, the low influx of Na^+ in NaCl^r mutant strain suggests the mutant strain saves a lot of energy by avoiding Na^+ efflux, that makes this strain more tolerant against NaCl (Page-Sharp et al., 1999).

Table 1: Growth (μh^{-1}) and heterocyst frequency (%) of wild-type and $NaCl^r$ mutant strain of *Anabaena variabilis* in presence and absence of NaCl (300 mM).

Sr. No.	Incubation conditions	Growth (μH^{-1})	Heterocyst frequency (%)
1.	Wild-type strain		
	Control (- NaCl)	0.032	6.0
	+ NaCl	0.0	0.0
2.	$NaCl^r$ mutant		
	Control (- NaCl)	0.033	8.0
	+ NaCl	0.036	13.0

In view of the above facts, it is reasonable to conclude that any substantial increase in NaCl concentration might adversely affect the population of diazotrophic cyanobacteria, which in turn, will affect the productivity of higher plants, especially rice in paddy fields. The application of $NaCl^r$ mutant strain of cyanobacteria, which can fix nitrogen at a higher pace, and can liberate most of the nitrogen as ammonia would be helpful in the reclamation of salt-affected soil and can be exploited to improve the agricultural economy of the developing countries.

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