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# Identification of nitrogen-fixing and salt-resistant cyanobacteria *Nostoc calcicola* isolated from the rhizosphere of cotton in Uzbekistan

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## ABSTRACT

At the concentration of NaCl at 200 mM, the nitrogen-fixing activity of *N. calcicola* insignificantly dropped against control and reached 88.2 nmol $C_2H_4$ flask/hour. After seven days of cultivation at the concentration of tryptophan; 3 and 5 mg/ml the synthesis of indole-3-acetic acid (IAA) in *N. calcicola* N 25 corresponded to the values 50,45; 97,5 and 210 mg/l.



## KEYWORDS

*Nostoc calcicola*;
Salt resistance;
Nitrogen fixation;
Indole-3-acetic acid (IAA).

## INTRODUCTION

One of the important factors of the increase of soil fertility is the biological fixation of the atmospheric nitrogen. The most important in this process is the cyanobacteria (blue-green algae), which, unlike the heterotrophic nitrogen fixers do not require nitrogen of the ready organic matter for the assimilation of the molecular nitrogen; instead, they themselves add it to the soil. For instance, for the soils of the moderate zone, the annual production of nitrogen fixing blue-green algae is assessed at 20-577 kg/ha (in dry weight). From the viewpoint of applied use they are technological, which includes cheap mediums for cultivation (absence of organic matter and sources of mineral nitrogen) and a quick accumulation of biomass even in extensive crops that do not require expensive equipment. Therefore, attention to this group of organisms in the practical aspect is concentrated on the study of their activity in the increase of soil fertility and crop yields of agricultural plants, and a possibility of producing active biopreparations on their basis[1-7].

The goal of our studies is the identification of nitrogen-fixing cyanobacteria of the genus *Nostoc* isolated from the rhizosphere of cotton

## MATERIALS AND METHODS

### Microorganisms

Cyanobacterial strains of our culture collection were selected for the present study. The strains *Nostoc calcicola* N 25 were previously isolated from the rhizosphere of cotton, which growing in salt-affected soils from Syrdaryo Province in Uzbekistan. Classification of the isolates was done by classical methods.

### Cultivation

The medium used to grow the strains was nitrogen-free "M" medium with following chemical ingredients (g/l): MgSO4 · 7H2O - 0.25; CaCl2 · 2H2O - 0.0238;

Na3C6H5O7 · 5.5H2O – 0.165; K2HPO4 – 0.04; trace elements - 1ml/l: (FeCl3 · 6 H2O - 0.002; ZnSO4 · 7 H2O - 0.222; CuSO4 · 5 H2O - 0.079; MnCl2 · 4 H2O - 1.81; Na2MoO4 · 2H2O - 0.03; H3BO3 – 2.80).

### Resistance of bacterial growth to salinity

For the determination of bacterial growth to salinity, the cultures with the titer $4,8x10^8$ cells/ml were grown on the agarized medium "M", containing a range of different concentrations of NaCl from 100 to 800 mM. The cultures were grown at 28ºC and growth of cultures was observed during 8-10 days.

### Nitrogenase assay in axenic cultures

Nitrogenase assay in axenic cultures Nitrogenase (nitrogen-fixing) activity was estiated by the acetylene-reductase activity (ARA) assay described by Hardy in cultures grown in 10 mL penicillin vials containing 4 mL of mineral medium "M"[8]. After 7 days of growth at the temperature 28ºC, the vials were tightly sealed with rubber caps and acetylene was injected into the headspace with a syringe to the final concentration of 10% (v/v). After 1 h of incubation with acetylene, concentration of ethylene in the gaseous phase was measured using a 'LHM-80' gas chromatograph (USSR). The acetylene-reductase activity of the cultures was expressed as nomoles C2H4/flask/ hour

### Indole-3-acetic acid (IAA) production in the strains

Cyanobacteria strains were inoculated in a respective medium with tryptophan (1, 3, and 5 mg/ml) or without tryptophan incubated at 28ºC for 7 days. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with drops of orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 10 ml 0.5 M $FeCl_3$)[9]. Development of the pink color indicates IAA production. O.D was read 530 nm using Spectronic 200. The level of IAA produced was estimated by a standard IAA (Serva) graph.

## RESULTS AND DISCUSSION

### Identification of local cyanobacteria *Nostoc calcicola*

Local strains of cyanobacteria of the genus *Nostoc* were isolated from the rhizosphere of cotton. The studied cyanobacteria of the genus *Nostoc* were algologically and bacterially purified and identified using the classical methods to the species *N calcicola* N 25. *N. calcicola* N 25 colonies are mucous, slightly diffused and of indefinite form. The vagina mainly unclear, more notable only at the periphery, colorless. Trichomes of *N. calcicola* №25 nearby 3,5-4 microns of width, heterocysts almost spherical, 4-5 microns in diameter. Spores spherical, 3-4 microns of width with a smooth yellow cover.

These signs are very valuable, when various cyanobacteria species of the genus *Nostoc* are isolated from numerous collected specimens; however, it is quite possible that heterocysts and spores may not form in cultured strains in response to the presence of nutrients in the habitat. Besides, biometric characteristics as heterocysts and akinetes of the cells of isolated blue-green algae may differ from natural specimens. For example, the analysis of the nucleotide sequences of the gene 16S rRNA showed that the *Nodularia* were similar to *Nostoc, Aphanizomenon* and *Anabaena*. It is necessary to note that the new genus *Spirirestis rafaelensis* has common morphological traits with the *Scytonemaceae* and *Microchaetaceae*; however, at the molecular level this genus is closely related with the *Microchaetacea*[10].

Currently, the phylogenetic analysis on the basis 16S rRNA nucleotide sequence is widely used as the instrument for bacterial taxonomy in general, and the advantages of this method were confirmed by many studies[11-13]. We have previously found a high genetic identity of the nucleotide sequence of 16S rRNA gene of the strain *N. calcicola* Uzb2 with other species of the cyanobacteria from the genus *Nostoc*. A comparative BLAST showed that the studied nucleotide sequence of the gene 16 S rRNA *N. calcicola* Uzb2 was homologous by 97 and 98% wuth the known strains of *Nostoc calcicola* HM573461.1 and *Nostoc calcicola* AM711529.1. This indicates that the local strain isolated by us by such morphological traits as the presence of akinetes (spores) and heterocysts and by the data of the molecular-genetic analysis belongs to the species *Nostoc calcicola* Uzb2[14].

### Growth and development of cyanobacteria *N. calcicola* N 25 at different stages of salinity

It is known that microphotosynthetics - filamen-

tous cyanobacteria and mobile diatomic algae adapted to sodium chloride and high alkalinity -– are active producers of the organic matter in saline soils together with higher plants. The aggregate biomass of cyanobacteria trichomes in saline soils exceeded 400 μg/g of soil, which creates a biogenic structure of the soil. The deficit of nitrogen containing organic substances in saline soils is partly compensated by the biomass of the cyanobacteria growth as soil films[1-4,7,15-18].

The further study of the effect of different concentrations of NaCl from 100 to 800 mM on the growth and development of *N. calcicola* N 25 showed that the salinity from 100 to 500 mM did not significantly affect the growth and development of cyanobacteria. It is noteworthy that the concentration of salt to 500 mM is not critical for the normal growth and development of cyanobacteria *N. calcicola* N 25. So, *N. calcicola* N 25 grows and develops in the medium containing 500 mM of NaCl and the titer of cells was 3,7 ’’’ $10^7$ cells/ml; at 800 mM of salt, the titer of cells dropped to 1,2’’’$10^7$ cells/ml (Figure 1). The growth of cyanobacteria in saline conditions is predetermined by the osmotic binding of water and a specific effect of ions on the protoplasm. A high concentration of salts binds water so that it becomes less accessible for the bacteria cells as the concentration of salts grows. It is known that osmoprotectants (proline, glycine-betaine, alanin-betaine) can be intensively synthesized and accumulated in the cells of gram-negative bacteria (cyanobacteria) inhabiting the soils containing increased concentrations of salts. Osmoprotectants can protect the bacterial cells and a plant in general from the salt stress, affecting the turgorness of bacterial cells[19,20].

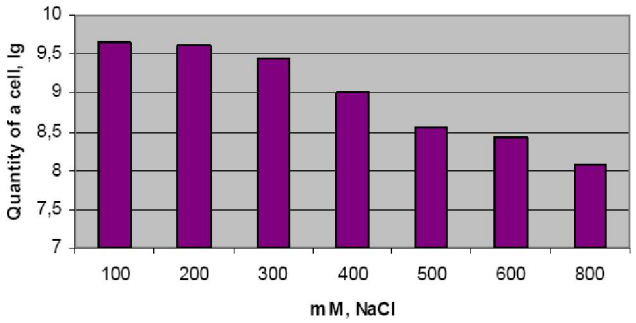


**Figure 1 : Growth and development of *N. calcicola* N 25 at different concentrations of NaCl.**

## Nitrogen fixing activity of cyanobacteria *N. calcicola* N 25 under conditions of salinity

The nitrogen fixing activity of the cyanobacteria *N. calcicola* N 25 was studied to identify the physiological state of the cyanobacteria *N. calcicola* N 25 under the salt stress. At the concentration of salt at 200 mM, the nitrogen fixing activity of the cyanobacteria *N. calcicola* N 25 insignificantly dropped compared to the control and reached 88.2 nmol $C_2H_4$ flask/hour. Experimental data show that the increase in the concentration of NaCl to 800 mM in the cultivation medium leads to the inhibition of activity of the nitrogenase by 36% (Figure 2).

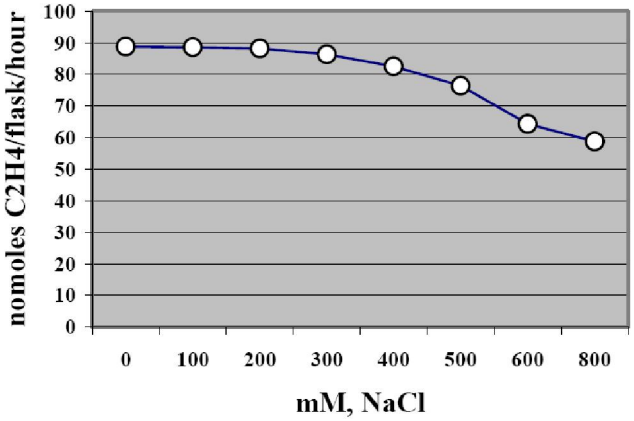


**Figure 2 : Nitrogen-fixing activity in cyanobacteria *Nostoc calcicola* (Uzb2) under conditions of salinity.**

The soil cyanobacteria in extreme conditions show a number of abilities, including enduring low humidity, sharp fluctuations of temperature, salinity and a heavy insolation. Owing to these abilities, the cyanobacteria are adapted to stress conditions by changing morphological and physiological traits (smaller sizes in comparison with water forms of these same species, profuse formation of mucus). Nitrogen fixing cyanobacteria are a main group of soil microorganisms, which make a significant contribution to the fertility of the soil. These organisms play an important role in this ecosystem, providing the soil with fixed nitrogen. Nitrogen fixation in the sizes that are important for the fecundity of the soil is typical of only heterocyst forms of cyanobacteria (representatives of the orders *Nostocales* и *Stigonematales*)[15,16].

## The synthesis of indole-3-acetic acid (IAA) by salt-resistant local cyanobacteria *N. calcicola* N 25

Unlike other representatives of soil algae, the het-

erocyst blue-green algae are able to fix from the atmosphere not only $CO_2$ (photosynthesis), but also the molecular nitrogen (nitrogen fixation), but also to produce different biologically active compounds, which determines their important role in the formation of organic matter[1,2,21,22].

Literature data of most researches do not provide detailed information on the mechanism of the action of cyanobacteria participating in the stimulation of growth and at the increase of the productivity of plants[18,23].

Previously we conducted studies on the effect of the cultural suspension of different local cyanobacteria strains from the genera *Nostoc, Anabaena* and *Gloeotece*, and different concentrations of IAA solutions as the control of the germination of cotton seeds in laboratory conditions[24]. It was established that *N. calcicola* N 25 stimulated the energy of germination capacity and the strength of their growth at low concentrations (1:1000) by approximately 45-55%, while at high concentrations (1:10) it inhibited them. Similar results were obtained in the control variants with IAA. Probably, the positive effect of the effect of low concentration of the cultural suspension of nitrogen fixing cyanobacteria *Nostoc calcicola* N 25 can be explained by the production of biologically active substances (auxin).

In this connection, we conducted studies of different levels of IAA production by cyanobacteria *Nostoc calcicola* N 25 at different concentrations of tryptophan at 1, 3 and 5 mg/ml and without tryptophan. As is known, the amino acid tryptophan is the most important source substance for the biosynthesis of auxin. The IAA is the product of the metabolic way of L-tryptophan, which is synthesized by some microorganisms and the addition of this amino acid to bacterial cultures leads to a higher production of the IAA. The biosynthesis of the IAA without tryptophan as a precursor was shown mainly in plants; however, it appears that it is quite extraordinary in bacteria[25-27].

As the figure 3 shows, the synthesis of the IAA without tryptophan in the cyanobacteria *N. calcicola* N 25 after three days of cultivation was equal to 10, 20 mg/l, after seven days, 19,0 mg/l.

After seven days of cultivation at the concentration of tryptophan at 1; 3 and 5 mg/ml the synthesis of the IAA in *N. calcicola* N 25 corresponded to the values 50,45; 97,5 and 210 mg/l. It was noted that at the addition of 5 mg/ml of tryptophan to the cultivation medium the protection of the IAA by this culture increased 11-fold compared to the experiment without the addition of tryptophan. This corresponds to the data of other scientists showing that tryptophan is the precursor of the IAA synthesis in *Nostoc calcicola* N 25[25-27].

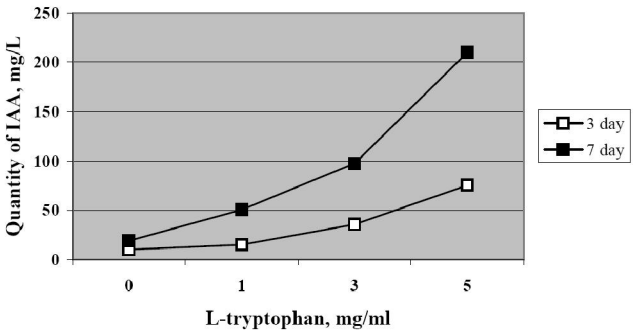


**Figure 3 : Production of the IAA by cyanobacteria *Nostoc calcicola* (Uzb2) after 3 and 7 days of cultivation both without and with L-tryptophan**

## CONCLUSION

Thus, the local salt-resistant cyanobacteria *N. calcicola* N 25 isolated and identified by us are not only the photosynthesizing nitrogen fixers, but also the biostimulators of growth and development of higher plants and other heterotrophic organisms, which enables the use of them in many Asian states (China, Japan, India, Vietnam, etc.) as biofertilizers. The role of cyanobacteria as accumulators of organic substances including the fixation of the molecular nitrogen, change of physical-chemical traits of the soil, stimulation of their microbiological activity is especially high in biocenoses developing in extreme conditions. Finally, isolated local nitrogen fixing cyanobacteria strains are the basis of fundamental and applied studies in agricultural biotechnologies and they supplement the collection of commercially valuable microalgae of Uzbekistan.

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