SALT-TOLERANT NITROGEN-FIXING BACTERIA PROMISING GROWTH OF PASTURE GRASSES ON SALINE SOILS

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Abstract

Pastures account for about 54% of the world's total land area. Currently, half of the world's pastures are degraded and salinized due to climate change and overexploitation. Salinization is one of the most serious abiotic stresses for plants. One way to address the problem is to mitigate salt stress by using beneficial microorganisms that can stimulate growth and improve plant immunity. The aim of the study was to isolate and identify salt-tolerant free-living nitrogen-fixing bacteria and study their effect on pasture grasses under salt stress. Soils were collected from the rhizosphere of pasture grasses growing on saline pastures in the southeast of Kazakhstan (Almaty region) and free-living nitrogen-fixing bacteria were isolated. Primary screening of bacteria was conducted and strains of salt-tolerant nitrogen-fixing bacteria were selected. Identification of the strains showed that the bacteria belong to different genera. The high stimulating effect of bacteria on the growth and development of cereal grasses growing on saline pastures has been shown. Thus, the use of salt-tolerant nitrogen-fixing bacteria helps plants resist salt stress and has great potential for maintaining their growth and development on saline soils.

Key words: *salinity, soil, salt-tolerant nitrogen-fixing bacteria, identification, salt stress, stimulation of plant growth*

1. INTRODUCTION

Pastures account for about 54% of the total land area. At the same time, they are important ecosystems that help maintain landscape biodiversity (Leroy et al., 2022). Grazing animals is highly profitable and determines employment and income for the rural population in many countries of the world (Wrobel, 2023). However, currently, half of the world's pastures are degraded and salinized due to the effects of climate change and overexploitation, which threatens the food security of many countries (Un Report, 2024). According to FAO (Food and Agriculture Organization of the United Nations), by 2050, the loss of arable land worldwide due to salinization will be 50% (FAO, 2022). Soil salinization is a serious abiotic stress that has a detrimental effect on plants (Abdel Latef et al., 2020). Salt stress negatively affects the nitrogen nutrition of plants and reduces the efficiency of soil nitrogen absorption by plants, which inhibits or completely stops their growth (Tarolli et al., 2024). It is well known that nitrogen is one of the main plant nutrients and is necessary for the biosynthesis of nucleic acids, enzymes, phytohormones, proteins and chlorophyll, its deficiency negatively affects the productivity of agricultural crops (Smirnova et al., 2023). In addition, the presence of high concentrations of salts in the soil reduces the use of nitrogen introduced with mineral fertilizers (Kamran et al., 2019).

Kazakhstan is a territory of pasture livestock farming, but most of the pasture soils are severely depleted. According to statistics, the total area of degraded pastures is more than 22.0 million hectares, of which saline pastures account for 31.3% (Buyanov, 2024). With salinization, pasture productivity decreases, soil structure deteriorates, and humus accumulation decreases. All these processes lead to the disappearance of valuable pasture forage crops, the main ones of which are cereal grasses (Pan et al., 2024). These grasses, when prevalent in pasture grass, have high nutritional value, promote healthy animal growth and increase their productivity. In addition, they help maintain the immune system and reduce animal susceptibility to diseases. In this regard, it is necessary to develop effective ways to increase the productivity of pasture lands.

One of the ways to improve pasture productivity is to use microorganisms that are present in the plant rhizosphere and have beneficial properties such as stimulating plant growth, improving nutrient uptake and mitigating salt stress. Rhizobacteria play an important role in improving plant growth through a wide range of mechanisms (Zhang et al., 2024). The ways in which rhizobacteria influence plant growth and development include different processes: increasing plant resistance to abiotic stresses, increasing nutrient uptake, producing plant growth regulators (phytohormones) and siderophores, producing volatile organic compounds and metabolites to prevent plant diseases (Brambilla et al., 2022; de Andrade et al., 2023). However, the main ones are biological nitrogen fixation, synthesis of phytohormones and increasing the availability of soil nutrients (Guimaraes et al., 2022).

One of the rhizobacteria are free-living nitrogen-fixing bacteria, which play a leading role in fixing nitrogen in the root zone of plants and enriching plants with biological nitrogen. The contribution of these bacteria to soil nitrogen enrichment is very high and reaches up to 300 kg N2/ha per year (Aasfar et al., 2021). Various strains of this group of bacteria have been isolated from the rhizosphere of agricultural crops, most often cereals such as wheat, barley, rice, meadow grasses, etc. (Guo et al., 2023). Some strains of these bacteria have been found to act as potential biological control agents and also secrete bioactive compounds that promote mineral absorption by enhancing root growth (Zhiyong et al., 2024). However, not all free-living nitrogen-fixing bacteria are able to develop and supply additional nitrogen to crops on saline soils. Therefore, the problem of finding effective strains capable of developing and fixing atmospheric nitrogen under salt stress conditions is very relevant. The aim of the study was to isolate and study salt-tolerant free-living nitrogen-fixing bacteria and study their effect on pasture grasses under salt stress conditions. Practical use of such bacteria in agriculture offers a promising approach to improving plant growth, especially under salt stress conditions.

2. MATERIALS AND METHODS

2.1 Objects of research

The objects of research were salt-tolerant free-living nitrogen-fixing bacteria. The bacteria were isolated in 2024 from the soils of saline pastures in Kazakhstan. The experiments were carried out using seeds of cereal grasses growing in natural grasslands of pastures in the Almaty region of Kazakhstan. The grass mixture included cereal grasses: timothy grass (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* L.) and perennial ryegrass (*Lolium perenne* L.), in a ratio of 1:1:1.

2.2 Methods

2.2.1 Collection of saline soil samples and their characteristics

Soil samples for bacterial isolation were collected in 2024 in the south-east of Kazakhstan in the Almaty region. Soil samples were collected from the rhizosphere of cereals growing on saline pastures in accordance with the State Standard (GOST 17.4.4.02-2017). The soil type is ordinary sierozem, the humus content is low - 0.8-1.1%, easily hydrolyzed nitrogen - 55.7 mg / kg of soil, mobile phosphorus - 20.5 mg / kg of soil and mobile potassium - 480 mg / kg of soil. Analysis of the aqueous extract of the soils showed that the pasture soil is highly saline, the salt content is 1.053%, the pH value is 9.2. According to the content of HCO_3^- ions, the pasture soils are alkaline.

2.2.2 Isolation of free-living nitrogen-fixing bacteria

To isolate nitrogen-fixing bacteria from soil samples, a dilution was prepared. For this, 10 g of soil was diluted with 90 ml of distilled sterile water and mixed on a shaker at 180 rpm for 2 hours. Then a series of dilutions was carried out by transferring 1 ml of the resulting suspension into a test tube containing 9 ml of sterilized water, etc. until a dilution of 10-8 was obtained. Microorganisms were seeded from dilutions of 10^{-5} and 10^{-6} on Petri dishes with nitrogen-free Ashby medium. Composition of Ashby medium (g / l): K_2HPO_4 - 0.2; $MgSO_4$ - 0.2; NaCl - 0.2; K_2SO_4 - 0.1; sucrose - 20.0; $CaCO_3$ - 5.0; distilled water - 1 L, pH 6.8. To isolate salt-tolerant bacteria, NaCl was added to the Ashby medium. The volume of the inoculum was 1 ml of suspension. Each dilution was sown in five replicates. The dishes were cultured at a temperature of 30 ° C for 5-7 days. The isolated nitrogen-fixing bacteria were cultured on an agar Ashby medium containing 5 % NaCl at a temperature of 30 ° C for 5-7 days. The purity of the cultures was periodically checked by microscopy with phase contrast and sowing on elective nutrient media.

2.2.3 Quantitative estimation of nitrogenase activity by bacteria

Nitrogenase activity was measured using the acetylene reduction assay (Senthilkumar et al., 2021). Bacteria were grown at 28 ° C and 180 rpm for 5 days on YEM medium. Then, the bacterial suspension with a concentration of 1×10^8 cells/ml was transferred to glass flasks (125 ml), from which 10 % of the air was removed and replaced with 10 % acetylene (vol/vol). Bacteria were incubated for 90 min. at 25° C. The amount of ethylene (C_2H_4) formed was measured using an Agilent Technology 7890B gas chromatograph (USA). The gas chromatograph was equipped with a flame ionization detector, the temperature was adjusted to 220 ° C, and the column thermostat was set at 60 ° C. The amount of ethylene formed was calculated by measuring the concentration of C_2H_4 in the sample, which was determined from the height of the sample peak relative to the C_2H_4 peak of the reference standard (Haskett et al., 2021).

2.2.4 Identification of bacteria by classical microbiological methods

To determine the species affiliation of active strains of microorganisms, their cultural-morphological, physiological and biochemical properties were studied in accordance with modern classification principles (Zvyagintsev, 1991; Ivshina, 2014). To determine the taxonomic position of bacteria and their identification, Bergey's Manual of Determinate Bacteriology was used (Holt, 1997).

2.2.5 Effect of bacteria on the growth of pasture grasses under salt stress conditions

To study the effect of bacteria on the growth of pasture grasses under soil salinity, grass seeds were sterilized and then inoculated with a bacterial suspension with a titer of 1×10^8 CFU/ml for 2 hours at a temperature of 23 ° C, at a rate of 5 ml of suspension per 1 g of seeds (Ashraf & Foolad, 2005). Then the seeds were sown in 250 ml vessels with saline soil (300 g) 1 g of seeds per vessel. For the experiments, highly saline soil with a total salt content in the aqueous extract of 1.061%, pH 9.0 was used. 30 days after the experiment, the plants were harvested, the roots and stems were dried to a constant weight, and their dry mass was determined. The experiments were carried out in a climatic chamber (Memmert HPP 750 Constant Climate Chamber, Germany). Daylight hours: 9 hours, temperature 25 ° C, illumination: cold white light - 6500 K, warm light 2700 K; night mode - 15 hours; temperature 18 ° C, humidity – 65 %. The studies were carried out in five replications.

2.2.6 Statistical analysis

The statistical significance of the obtained results was analyzed using the STATISTICA 10.0, ver. 6.0 software package. Differences were considered significant at $p <0.05$, and the values are presented as the mean (M) ± standard error of the mean (±SEM).

3. RESULTS

Large pasture grass plants growing on saline soils were selected for the isolation of bacteria. The rhizosphere soil was collected in May-July during the vegetation period of plant growth. The roots of the plants with a 20 cm^2 diameter soil lump were shaken vigorously for 10 min, maintaining the integrity of the roots. After that, the rhizosphere soil firmly adhering to the roots was collected; a total of 68 rhizosphere soil samples were collected. Bacteria were isolated in laboratory conditions on nitrogen-free Ashby medium with 5% NaCl to create salinity. As a result of the work, 57 isolates of free-living nitrogen-fixing bacteria capable of active growth on Ashby medium with high salinity were isolated.

In order to determine the taxonomic position of the isolated nitrogen-fixing bacteria, their main cultural, morphological and biochemical features were studied. It was found that the isolated bacteria mainly belong to the genera *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Agrobacterium*. It was found that more than 50% of the total number of free-living nitrogen-fixing bacteria were bacteria belonging to three genera *Azotobacter, Pseudomonas* and *Agrobacterium*, and 35% of them were the genus *Azotobacter*.

Next, the bacteria were screened for their ability to grow under salt stress. To create salt stress, NaCl was added to Ashby's medium in different concentrations: 100, 250 and 500 mM. Table 1 shows the data on the 10 most salt-tolerant isolates.

	Concentration NaCl (mM)		
Isolates	**100**	**250**	**500**
Az3/2	+++	+	-
Az3/9	++	++	+
Az3/23	+++	++	+
Az3/28	++++	+++	++
Az3/29	++++	+++	++
Azp6	++++	+++	++
Az22	++++	+++	++
Az24	+++	++	++
Az32	++	+	-
Az38	++	+	-

Note: Qualitative assessment: - indicates absence of the growth; indicates presence of the growth; additional + indicates intensity of the growth exhibited by the isolates

Table 1. Tolerance of isolates of the nitrogen-fixing bacteria to salt stress

It follows from the data in Table 1 that not all isolates had the same salt tolerance. Thus, if at a NaCl concentration in the medium of 100 mM all isolates grew, then at a concentration of 500 mM NaCl only seven did: Az3/9, Az3/23, Az3/28, Az3/29, Azp6, Az22, Az24. These seven isolates were selected for further work as the most salt-tolerant. One of the important indicators in the practical application of free-living nitrogen-fixing bacteria is their ability to fix molecular nitrogen from the atmosphere on saline soils. For this purpose, the nitrogenase activity of bacteria and their ability to accumulate biomass under high salt stress were studied. For this purpose, the isolates were grown on Ashby liquid medium with a NaCl concentration of 500 mM. The table presents data on the accumulation of biomass and their nitrogenase activity of bacteria that showed the highest results.

Isolates	**Dry biomass (g/l)**	**Nitrogenase activity (µmole C_2H_4/ml/h)**
Az3/9	2.52	5.41*
Az3/23	1.15	3.56
Az3/28	1.81	4.19
Az3/29	2.61	4.94*
Azp6	2.74	5.27
Az22	2.43	4.71
Az24	1.47	4.65*

Note: Values are means ± standard error of the mean (± SEM), $p < 0.05$; *$p < 0.01$; n = 5

Table 2. Biomass and nitrogenase activity of isolates of the nitrogen-fixing bacteria

It follows from the data in Table 2 that the studied strains actively fix atmospheric nitrogen at a high degree of salinity, as evidenced by high rates of nitrogenase activity and biomass production, which varied from 1.15 to 2.74 g / L. Three strains Az3 / 28, Az3 / 29, Azp6 were characterized by the highest accumulation of biomass and nitrogenase activity. These strains were selected as salt-tolerant bacteria and used for further work.

To set up experiments on the effect of salt-tolerant nitrogen-fixing bacteria on the growth and development of cereal grasses, highly saline soil was used (salt content of aqueous extract 1.051%, pH 9.2). Cereal seeds without inoculation with bacteria were used as a control. The obtained data are presented in Figure 1.

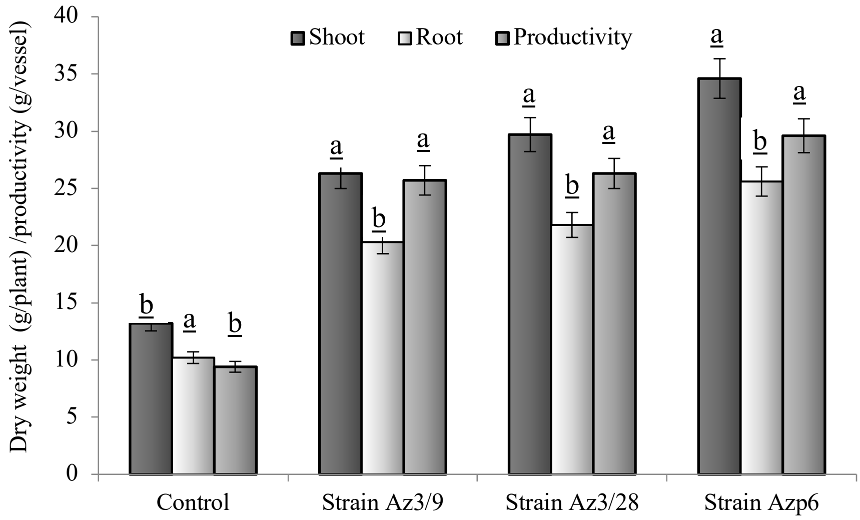

Fig. 1. Effect of salt-tolerant nitrogen-fixing bacteria on the growth of cereal grasses

Columns represent mean values while bars represent standard deviation (n = 5). Different letters show statistically significant different values ($p < 0.05$) between strains.

The experiments showed that inoculation of cereal grass seeds with salt-tolerant nitrogen-fixing bacteria stimulated plant growth and development under high salt stress (Figure 1). At the same time, the dry weight of plant stems increased on average by 2.0-2.6 times, roots - by 3.2-4.2 times, and the yield of green mass of plants - by 3.0-3.3 times compared to the control. Also, seed inoculation treatment increased the length of plant roots by 3.2-4.5 times, which indicates the ecological adaptation of plants to soil salinity. It can be said that salt-tolerant nitrogen-fixing bacteria stimulate the growth and development of cereal grasses and partially relieve stress on saline soils.

4. DISCUSSION

Grasslands account for about 54% of the total land area and half of the world's natural grasslands are degraded, posing a threat to biosecurity [Hou et. al, 2022]. The United Nations Convention to Combat Desertification (UNCCD) has warned that one sixth of the world's food supplies are at risk due to rangeland degradation [UNCCD, 2024].

Due to soil salinization, pasture productivity is sharply reduced and restoring their fertility faces many challenges (Atta et al., 2023). Salinity is a major abiotic factor that inhibits plant growth. Salinity stress causes plant damage due to excessive accumulation of soluble ions (Na+, Ca+, K+, Mg2+) in the root zone (Atta et al., 2022). For farming on saline soils, salt-tolerant varieties are bred using traditional selection or genetic modification of plants. However, these methods do not give the desired results and are not sustainable. In recent years, more and more attention has been paid to research on mitigating the effects of salinization by using rhizosphere bacteria that promote plant growth, one of which is free-living nitrogen-fixing bacteria.

A total of 68 soil samples were collected from the rhizosphere of pasture grasses growing on saline pastures in the southeast of Kazakhstan (Almaty region) and 57 isolates of free-living nitrogen-fixing bacteria capable of active growth on Ashby medium with high salinity were isolated. The taxonomic position of the isolated isolates was determined and it was found that the isolated bacteria mainly belonged to the genera Azotobacter, Azospirillum, Bacillus, Pseudomonas, Agrobacterium, with more than 50% of the total number of free-living nitrogen-fixing bacteria belonging to the three genera Azotobacter, Pseudomonas and Agrobacterium, with 35% of them belonging to the genus Azotobacter. These results are inconsistent with the data of a number of authors who found that Pseudomonas isolates isolated from saline soils were the most numerous groups and accounted for 60-80% (Li et al., 2021; Koirala et al., 2025).

The conducted primary screening of bacteria based on salt tolerance allowed us to select salt-tolerant nitrogen-fixing bacteria. It was found that out of 57 isolated isolates, only seven were salt-tolerant Az3/9, Az3/23, Az3/28, Az3/29, Azp6, Az22, Az24 and only seven could grow at a concentration of 500 mM NaCl. These isolates were selected for further work as the most salt-tolerant. Similar results were obtained in the studies of other authors. Thus, Sharma et al. (2021) isolated 81 bacterial isolates from saline soils and only five of them were salt-tolerant. Other authors showed that out of 86 strains of potential nitrogen-fixing bacteria, only three strains were selected (Shi et al., 2023).

A very important property of bacteria is their ability to actively fix atmospheric nitrogen under high salinity. In our studies, we showed that the isolated bacteria are capable of fixing atmospheric nitrogen under high salt stress. This is evidenced by the indicators of nitrogenase activity and biomass production. It was found that the highest indicators were characterized by three strains Az3 / 28, Az3 / 29, Azp6. We also found that an increase in salinity reduces the ability of bacteria to fix nitrogen. Indirect evidence suggesting that salinity levels may affect nitrogen fixation is presented in the work of Liu et al. (2022).

A high stimulating effect of bacteria on the growth of cereal grasses growing on saline soils was established. At the same time, the dry weight of stems, roots and the yield of green mass of meadow grasses increased by 3-4 times compared to the control. Also, seed inoculation treatment increased the length of plant roots by up to 4.5 times, which indicates the ecological adaptation of grasses to soil salinity. It can be said that salt-tolerant nitrogen-fixing bacteria stimulate the growth and development of cereal grasses and partially relieve stress on saline soils. Similar studies on the effect of salt-tolerant nitrogen-fixing bacteria on the growth of meadow grasses have not been found. However, there are many publications that indicate a positive effect of rhizosphere nitrogen-fixing bacteria on the growth of other plants and their mitigation of salt stress (Gul et al., 2023; AbuQamar et al., 2024; Agbodjato & Babalola, 2024).

5. CONCLUSIONS

In our study, we collected soil from the rhizosphere of cereal grasses growing on saline pastures in the Almaty region of Kazakhstan and isolated free-living nitrogen-fixing bacteria. They were identified to the genus level. It was found that the isolated bacteria belonged to different genera, but 50% of the total number of bacteria were three genera: Azotobacter, Pseudomonas and Agrobacterium, of which 35% were the genus Azotobacter. The salt tolerance of the bacteria and the ability to fix molecular nitrogen in the air under high salt stress were studied and three highly active strains were selected. Their high stimulating effect on the growth, development and productivity of cereal meadow grasses growing on saline soils was established.

In the future, we plan to conduct a more detailed study of the selected bacterial strains and investigate their ability to synthesize phytohormones, ACC deaminase, and other secondary metabolites to support plant growth and increase the availability of nutrients under salinity. The results of our studies allow us to conclude that the use of salt-tolerant nitrogen-fixing bacteria on saline soils helps plants resist salt stress and they have great potential for sustainable agriculture on saline soils.

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