RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
55 (3) 810-824, 2023
http://doi.org/10.54910/sabrao2023.55.3.17
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

USING SALT-TOLERANT RHIZOBIA TO IMPROVE THE SOYBEAN (*GLYCINE MAX*) RESILIENCE TO SALINITY

I. SMIRNOVA[*], A. SADANOV, G. BAIMAKHANOVA, E. FAIZULINA, and L. TATARKINA

Research and Production Center for Microbiology and Virology LLC, Republic of Kazakhstan
*Corresponding author's emails: iesmirnova@mail.ru
Email addresses of co-authors: a.sadanov@inbox.ru, bgulb@mail.ru, elmira_f@mail.ru, tatalora@mail.ru

SUMMARY

Soybean (*Glycine max* [L.] Merr.) is an economically important oilseed crop with an annual increase in growing grain demand. Soybean is a moderately salt-tolerant crop; however, salt stress conditions can affect its growth and yield-related traits and, eventually, reduce productivity. In saline soils, one of the techniques to increase soybean productivity is to use rhizobia inoculation. Although, using industrial rhizobia-based biofertilizers is often ineffective due to their lack of adaptability to salinity. Injecting soybeans with salt-tolerant and growth-promoting rhizobia helps mitigate the effects of salt stress harmful to crop plants. The recent study sought to isolate local strains of salt-tolerant rhizobia, studying its ability to increase soybean tolerance to salt stress conditions. Twenty-four local salt-tolerant rhizobium isolates underwent isolation from root nodules of soybean grown on saline soils. Studying their basic morphological and biochemical characteristics and ability to withstand salt stress led to the final selection of five salt-tolerant strains. The rhizobium strains were able to synthesize metabolites that stimulate growth and help reduce salt stress in plants. The study of rhizobia nodulation ability under saline conditions resulted in selecting the three most efficient strains from the *Bradyrhizobium japonicum* species. Inoculation of soybean seeds with salt-tolerant rhizobia proved to mitigate the adverse effects of salinity on plant growth by increasing the root size and the number of nodules in the roots. Thus, the study establishes that inoculation of soybean seeds with local salt-tolerant rhizobia enhances soybean tolerance to salt stress and improves crop growth and adaptation to soil salinity. Using isolated local strains of salt-tolerant rhizobia will help provide a key and environmentally friendly approach to solving the problem of salt stress for sustainable agriculture.

Keywords: Soybean (*Glycine max* [L.] Merr.), salt-tolerant rhizobia, inoculation, salt stress conditions, salt tolerance, growth and yield traits

Communicating Editor: Dr. Kamile Ulukapi

Manuscript received: February 10, 2023; Accepted: April 15, 2023.

Citation: Smirnova I, Sadanov A, Baimakhanova G, Faizulina E, Tatarkina L (2023). Using salt-tolerant rhizobia to improve the soybean (*Glycine max*) Resilience to salinity. *SABRAO J. Breed. Genet.* 55(3): 810-824. http://doi.org/10.54910/sabrao2023.55.3.17.

Key findings: Soybean inoculation with salt-tolerant rhizobia significantly reduced the salt stress effects on the plants. Substantial differences also showed between the variants with inoculation and the control (untreated). Inoculating leads to a considerable increase in root nodules, nitrogen fixation, plant growth, and stimulation of root development, which is proof of the ecological adaptation of soybean plants to soil salinity.

INTRODUCTION

Soybean is a unique and valuable crop globally, with an increasing grain demand every year. The highest demand for soybean is due to the following: 1) soybean seed is a richer source of protein; 2) it fulfills the protein requirements in countries with economically inaccessible meat and dairy products, making soy a unique product; and 3) soybean cultivation helps solve the food and feed protein deficiency issues (Wijewardana *et al*., 2019). Soybean is a moderately salt-tolerant crop, and soil salinity is a chief limitation to their production in many regions worldwide (Ilangumaran *et al*., 2021; Lestari *et al*., 2022). Crop exposure to salt stress results in slower growth, increased leaf chlorosis, decreased biomass accumulation and protein content, and inhibiting the formation of nodules and nitrogen fixation, which eventually causes a significant decrease in crop yield (Adsul *et al*., 2016; Otie *et al*., 2021). For instance, soil salinity ranges from 0.1% to 0.4% could reduce the soybean yield by 50% (Ren *et al*., 2016; Alam *et al*., 2019).

In the present era, soil salinization is escalating and becoming more severe (United Nations, 2022). The area of saline irrigated land varies from 20% to 25% and continues to expand at an annual rate of 1–2 million ha around the world, making soils unsuitable for cultivation (Devkota *et al.,* 2022). Expected land degradation may reach 50% by 2050 (Hossain, 2019). Soil degradation, in turn, threatens food security; therefore, sustainable research on mitigating soil salinity is a highly contemporary issue.

Soil salinization has several causes, with climate change as the most common cause. Generally, the higher air temperatures exaggerate soil evaporation and aggravate soil salinization (Asad *et al*., 2021). Another reason is the less freshwater availability and the use of treated wastewater and low-salinity water for irrigation (Mohanavelu *et al*., 2021). In addition, the decrease in freshwater supply leads to drip and sprinkle irrigation that lessens the leaching of accumulated salts in soils (Minhas *et al*., 2020). Saline soils significantly impact the environment and act as intense abiotic stress to plants (Chele *et al*., 2021). Reports said that up to 70% of crop yields in the future could be lost due to soil salinity (Nawaz *et al*., 2020).

In Kazakhstan, the soybean crop is mainly a source of protein for fodder and raw material for food production (Qin *et al*., 2022). Despite a larger area for soybean cultivation in Kazakhstan, its yield is still low, not exceeding 1.1 to 1.2 t/ha (Didorenko, 2021). Soil salinity is one of the major causes of low soybean yield in Kazakhstan. The main shortfall (83%) in soybean crop yield of the country is in the Almaty region, with soils marked by high salinity and low nitrogen and phosphorus content (Iorgansky *et al*., 2017).

Developing crops capable of tolerating salinity stress progresses to avoid yield losses due to soil salinity. Conventional breeding and genetic engineering are crucially advanced technologies for developing crop cultivars with improved salt tolerance. However, using genetically modified salt-tolerant soybean varieties has yet to see a positive impact due to various factors (Saradadevi *et al*., 2021). Producing salt-tolerant varieties through traditional breeding is also a long process and laborious (Voss-Fels *et al*., 2019). In this connection, the most promising is to use salt-tolerant rhizobia that can actively form the root nodules and stimulate plant growth to reduce the salt stress effects on soybean (Ali *et al*., 2022).

Increasing the productivity of soybeans in saline soils results from using chemical nitrogen and phosphorus fertilizers. These fertilizers enhance soybeans yet harm the environment (Guignard *et al.*, 2017). An alternative to increase soybean productivity is utilizing genetically modified salt-tolerant varieties. However, less good results come from this (You *et al.*, 2022). Conventional breeding is also a strategy to improve soybean salt tolerance, but its effectiveness is low (Yang *et al.*, 2020). The most promising solution to this problem is employing a biological method that inoculates soybean seeds with rhizobia. Although, the imported biofertilizers are often ineffective in the saline soils of Kazakhstan. It is attributable to biofertilizer rhizobia not forming nodules on soybean roots due to the lack of rhizobia strain adaptation to salty soil conditions (Smirnova *et al*., 2022).

The presented study sought to isolate the local strains of salt-tolerant rhizobia, studying their ability to increase the salinity tolerance in soybean plants. The research isolated local salt-tolerant rhizobia strains, assessing their ability to form nodules with active nitrogen fixation on saline soils. The isolated effective strains that promote soybean plant growth and reduce the salt stress effects constitute the scientific novelty of the presented study. Furthermore, the isolated rhizobia strains contribute to better adaptation of soybean plants to saline soils. The practical significance of this study displays that applying local salt-tolerant rhizobia will increase soybean resistance to salt stress, allow soybean cultivation on saline soils, and increase its yield.

MATERIALS AND METHODS

Breeding material and procedure

The research on agrochemical properties of soils transpired at the Kazakh Research Institute of Soil Science and Agrochemistry, named after U.U. Uspanov LLP, Chemical Analysis Laboratory (Kazakhstan, Almaty). The Eureka variety of soybean (*Glycine max* [L.] Merr.) had rhizobium isolated from root nodules. During the flowering phase in 2021, the collected and selected soybean plants have well-formed and visible nodules. Selecting two saline fields in the Almaty region of Kazakhstan ensued for plant collection. These fields were medium saline (N 40°50.208; E 68°30.112) and heavily saline (N 40°50.120; E 68°29.165) at 265 masl. The region is characteristic of a sharply continental climate, with average annual precipitation of up to 300 mm. The soil was an ordinary gray soil with a low humus content (0.95%–1.1%), easily hydrolyzed nitrogen (58.2 mg/kg soil), mobile phosphorus (21.4 mg/kg soil), and mobile potassium (460 mg/kg soil). In these two fields, soil (at 10–20 cm depth) analysis consisted of basic agrochemical properties. Table 1 presents the analysis of soil aqueous extract.

The first field comprised of the medium-saline soil, with a salt content of 0.198% ± 0.01% – 0.389% ± 0.02% and a pH value of 8.9 ± 0.01. The second site was highly saline with a salt content of 0.515% ± 0.01% – 1.051% ± 0.06% and a pH value of 9.2 ± 0.02 (Table 1). Based on HCO_3^- ion content, the fields where the harvest of soybean plants occurred belong to slightly alkaline soils (0.015% – 0.02%). The conducted experiments on seeds of the soybean cultivar Eureka used a soybean cultivar that is medium maturing, drought tolerant, recommended for cultivation in the Almaty region of Kazakhstan, and most widely used by farmers. The seeds came from the Department of Fodder, Oil Crops, and Corn, Kazakh Research Institute of Agriculture and Plant Growing LLC, District Karasai, Almaty region, Kazakhstan.

Isolation of rhizobia

For the identification of salt-tolerant rhizobium isolates, during the flowering phase, collecting a total of 75 soybean plants progressed from the fields of the Almaty region of Kazakhstan with moderate to severe soil salinity. The selected plants for rhizobia isolation were healthy, with well-developed roots and large nodules. After separating the nodules from the rhizomes, their surface sterilization included immersion in 70% ethanol for 2 min; then, in a 2% sodium hypochlorite solution (NaOCl) for 5 min, and then washed with sterile distilled water until the smell disappeared. Later, crushing the nodules and transferring them to

Table 1. Water extract in % on absolutely dry soil in the phases of soybean growth.

Sate	Sum salts (%)	Alkalinity (mM [eq] /100 g soil)	Ionic concentration (mM (eq)/100 g soil)					
		$HCO3^{-}$	Cl^{-}	$SO4^{2-}$	Ca^{2+}	Mg^{+}	Na^{+}	K^{+}
Before sowing (April)								
Medium saline soil	0.198±00.01	0.02-0.03	0.007-0.008	0.119-0.120	0.025-0.027	0.014-0.015	0.014-0.016	0.002-0.003
Heavily saline soil	0.515±00.01	0.,019-0.02	0.024-0.026	0.335-0.338	0.073-0.074	0.035-0.036	0.032-0.033	0.003-0.005
Soybean seedlings (May)								
Medium saline soil	0.269±00.01	0.018-0.02	0.015-0.017	0.162-0.164	0.036-0.038	0.015-0.016	0.026-0.028	0.004-0.006
Heavily saline soil	0.642±00.02	0.018-0.019	0.036-0.038	0.388-0.392	0.094-0.096	0.025-0.027	0.062-0.063	0.007-0.009
Soybean bloom (July)								
Medium saline soil	0.282±00.01	0.018-0.019	0.009-0.01	0.173-0.175	0.037-0.038	0.016-0.017	0.021-0.023	0.006-0.007
Heavily saline soil	0.781±00.02	0.015-0.017	0.034-0.035	0.515-0.517	0.098-0.099	0.047-0.049	0.062-0.063	0.008-0.009
Soybean ripening (September)								
Medium saline soil	0.350±00.01	0.016-0.018	0.015-0.017	0.228-0.230	0.045-0.047	0.023-0.024	0.029-0.031	0.006-0.008
Heavily saline soil	0.829±00.02	0.015-0.016	0.057-0.06	0.541-0.543	0.069-0.072	0.070-0.071	0.084-0.086	0.005-0.006
After harvest (October)								
Medium saline soil	0.389±0.02	0.015-0.017	0.015-0.016	0.263-0.264	0.059-0.061	0.024-0.025	0.024-0.025	0.004-0.006
Heavily saline soil	1.051±0.06	0.015-0.016	0.079-0.081	0.663-0.665	0.072-0.075	0.073-0.074	0.091-0.096	0.006-0.008

Petri dishes contained a medium with yeast extract mannitol (YEM) with added 10% NaCl solution and smeared on the surface of the medium with a spatula. The same spatula used in the subsequent seeding of three more dishes served to obtain separate colonies (Albdaiwi *et al.*, 2019). The dishes with isolates followed cultivation at 28 °C for seven to nine days. Growing on the YEM medium, rhizobia formed colorless or milky-white mucilaginous colonies.

Rhizobium isolates screening for salinity tolerance

Rhizobium isolates tested for tolerance to salt stress had different concentration levels of NaCl according to a pre-developed protocol. Growing the isolates in liquid Iswaran medium with the addition of NaCl (100, 250, and 500 mM) took five days at 28 °C and 180 rpm. The composition of the liquid Iswaran medium included (g/l): sucrose - 10.0, K_2HPO_4 - 0.5, $MgSO_4 \times 7H_2O$ - 0.2, calcium gluconate - 1.5, $FeCl_3$ - 0.01, table water - 1,000 ml, and pH - 6.8–7.0. Bacterial growth recording was by optical density at 540 nm on a spectrophotometer (PD-303, Apel, Japan). Later, the growth of isolates gained evaluation, where the minus (−) sign corresponds to a complete absence of growth, plus (+) sign for the presence of growth, and the addition of a plus (+) indicates the growth intensity on the saline medium. Experiments were in five-fold replications.

Quantitative estimation of nitrogenase activity by rhizobia

Nitrogenase activity measurement used the acetylene reduction assay. The rhizobia were grown at 28 °C and 180 rpm on the YEM medium for five days. The bacterial suspension transfer proceeded at a concentration of 1×10^8 c/ml in glass vials (125 ml), from which the removal of 10% of the air got replaced with 10% acetylene (vol/vol). The rhizobia incubation succeeded for 90 min at 25 °C. Measuring the amount of ethylene production (C_2H_4) used an Agilent Technology 7890B gas chromatograph (USA). The gas chromatograph, equipped with a flame isolation detector, brought the temperature to 220 °C, with the thermostat column set to 60 °C. Calculating the amount of ethylene produced was by measuring the concentration of C_2H_4 in the sample, as determined by the height of the sample peak relative to the peak of reference standard C_2H_4 (Haskett *et al*., 2021).

Determination of 1-aminocyclopropane-1-carboxylic acid deaminase activity by rhizobia

Rhizobium isolates grown under 28 °C in BD Tryptic Soy Broth (Soybean-Casein Digest Medium, Sigma-Aldrich, Germany) helped determine the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. After 24 h of growth, the bacteria were centrifuged at 10,000 g for 5 min and then suspended in 5 ml of Dworkin and Foster's medium of the following composition (g/l): 4.0 - KH_2PO_4, 6.0 - Na_2HPO_4, 0.2 - $MgSO_4 \times 7H_2O$, 2.0 - glucose, 2.0 - gluconic acid, 2.0 - citric acid with trace elements (1 mg $FeSO_4 \times 7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO \times H_2O$, 124.6 mg $ZnSO \times 7H_2O$, 78.22 mg $CuSO_4 \times 5H_2O$, 10 mg MoO_3), pH 7.2, containing 5 mM/l^{-1} ACC as a sole source of nitrogen, then, incubated for 24 h at 28 °C. Determining the quantitative ACC deaminase activity of the isolates was spectrophotometrically (PD-303, Apel, Japan) by α-ketobutyrate at 540 nm and compared with standard α-ketobutyrate (Sigma-Aldrich, Germany) in the range of 0.1 to 1.0 μM. According to the standard curve, the unit of ACC deaminase activity was the amount of α-ketobutyrate produced in μM/mg $\text{protein}^{-1}/h^{-1}$. The analysis was in five-fold replications.

Determination of indole-3-acetic acid production by rhizobia

The identification of indole-3-acetic acid (IAA) by isolates made use of a gas chromatograph (Agilent Technology 7890B, USA), according to the protocol described by Hernández-León *et al*. (2015). The bacterial cultures added with tryptophan attained incubation under stirring (250 rpm) at 30 °C for 48 h. Comparing the retention times in the bacterial extracts with samples of pure standard IAA (Sigma, Germany) confirmed the identity of the IAA. Constructing an individual graduation curve helped estimate the amount of IAA produced by the strains. The definition of the IAA in different bacterial strains was in five-fold replications.

Identification of rhizobia

Rhizobia identification went on by sequencing variable fragment 16S rRNA gene on the Applied Biosystems™ 3500 DNA Analyzer platform. Amplification of DNA fragments ensued with standard primers (Winand *et al*., 2020) by 30 cycles in an Eppendorf ProS thermal cycler (Eppendorf, Hamburg, Germany, 2012) with Q5® Hot Start High-Fidelity 2X Master Mix. The search for reference sequences followed using the BLAST program (Basic Local Alignment Search Tool) in the Gene Bank International Database NCBI, USA (NCBI, 2020). Performing the phylogenetic analysis engaged the DNA Star Lasergene software with the ClustalW algorithm and NJ (neighbor-joining) method.

Study of rhizobia nodulation under salt stress

Rhizobia were grown on liquid Iswaran medium at 180 rpm and 28 °C for seven days. The surface of soybean seeds’ sterilization consisted of 1% sodium hypochlorite solution (NaOCl) for 5 min, with the seeds washed after with sterile distilled water until the odor disappeared (Abduhu *et al*. 2018). Before sowing, inoculation of seeds applied a bacterial suspension with a cell titer of 1×108 c/ml at 10 ml per 1 g of seeds for two hours at 23 °C, then sown into 500 ml growing vessels (three seeds per vessel). Sterilized vermiculite was the substrate for plant growth, and adding 250 L of water or NaCl solution with concentrations of 250 and 500 mM to each vessel occurred before sowing. For nourishment, the plants received watering once a week with a sterile solution of 1 g of water-soluble fertilizer (5-0-4, General Hydroponics, USA), 1 g of double superphosphate, and one L of water. The negative control was soybean seeds without rhizobia inoculation soaked in sterile water under the said conditions. The positive control was the seeds treated with rhizobia, but without adding NaCl. After 45 days of cultivation, the number of nodules, the dry weight of nodules and roots, and the nitrogen-fixing activity of nodules got determined, as described in Das and De (2018), using the acetylene reduction assay (Kaushal and Kaushal, 2015). The experiments materialized in an environmental chamber (Constant Climate Chamber Memmert HPP 750, Germany) under the following regime: light day - 9 h; temperature – 25 °C; illumination: cold white light - 6,500 K, warm light - 2,700 K; night mode - 15 h; temperature - 21 °C; and humidity - 65%. All experiments were in five replicates.

Study of soybean growth under salt stress by inoculation with salt-tolerant rhizobia

Applying the impact of salt-tolerant rhizobia on the development of soybean plants under salt stress began in growing vessels (5,000 ml) with highly saline soil. The soil type was ordinary gray. Agrochemical indicators were: humus - 1.1%, easily hydrolyzed nitrogen - 56.8 mg/kg soil, mobile phosphorus - 20.8 mg/kg soil, and mobile potassium - 457.1 mg/kg. The reaction of the soil was highly alkaline (pH - 9.2). Based on the previous procedure, growing rhizobia on liquid Iswaran medium was at 28 °C, 180 rpm for five days, with seeds of the soybean cultivar Eureka sterilized as described previously. Before sowing, inoculating the seeds with rhizobia suspension comprised a titer of 10^8 c/ml for two hours at 23 °C, at 5 ml per seed, and planting five seeds per vessel. After one week of cultivation, seedlings were thinned to three in each vessel. The negative control was seeds treated with sterile water. The experiments were in five-fold replications for 60 days. After 60 days, the number and dry weight of nodules and the dry weight of roots, shoots, and leaves were measured (drying at 70 °C until constant weight). The experiment had three repeats in an open area under conditions close to field environments.

Statistical analysis

All the recorded data underwent statistical analysis using the software package STATISTICA 10.0, var. 6. Tables and figures show the mean values (M) and standard errors of means (±SEM).

RESULTS

From soybean root nodules, isolation resulted in 24 potentially pure salt-tolerant bacterial isolates capable of growing on saline media. The isolates screened for resistance to salt stress received three different concentrations of NaCl (100, 250, and 500 mM). The experiments used liquid Iswaran medium. The data for the 10 most salt-tolerant isolates appear in Table 2, revealing that not every rhizobium isolate can withstand high salt concentrations. Therefore, the growth of the isolates differed markedly under study, with some isolates reduced and inhibited by salt. Almost all isolates grew well at 100 mM NaCl concentration in the medium, but their growth differs significantly. Four isolates, MA-1, MA-31, RH-18, and RH-22, showed very poor growth (+). These isolates showed growth inability even at 250 mM and 500 mM NaCl in the medium. Only five isolates, RH-3, RH-4, RH-6, RH-7, and RH-8, grew at 500 mM NaCl in the medium, and these isolates exhibited the highest salt tolerance.

Studying the cultural and morphological characteristics of the selected rhizobium isolates also commenced. Bacteria formed colorless or milky-white mucilaginous colonies when grown on a maze medium. In test tubes on this medium, rhizobia formed transparent mucilaginous, dripping downward stippling colonies. Examining bacterial cell morphology revealed that all the identified isolates were Gram-negative, unable to form spores, and had a bacilliform cell shape. The bacterial cells appeared to be small and

Table 2. Screening for salinity tolerance of the rhizobium isolates.

Strain	Concentration NaCl (mM)		
	100	250	500
MA-1	+	-	-
MA-31	+	-	-
RH-2	++	+	-
RH-3	+++	+++	++
RH-4	++++	+++	++
RH-6	++++	++++	+++
RH-7	++++	++++	+++
RH-8	++++	++++	+++
RH-18	+	-	-
RH-22	+	-	-

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

Table 3. PGP activity of rhizobium isolates.

Rhizobia	Nitrogenase activity (µmole C_2H_4/ml/h)	ACC-deaminase (mM α-ketobutyrate/mg^{-1} protein/h^{-1})	IAA (µg/ml^{-1})
RH-3	5.25±0.1*	0.41±0.01*	38.25±1.1
RH-4	6,45±0.2*	0.43±0.02*	42.45±1.1
RH-6	6.71±0.1*	0.54±0.02*	46.17±1.6
RH-7	6.74±0.2*	0.56±0.01*	48.42±1.2
RH-8	6.73±0.1*	0.51±0.01*	47.37±1.1

Note: Values are means ± standard error of the mean (±SEM), n = 5. The standard deviation is given, indicating significant differences based on the *Student-Newman-Keuls* test (SNK-test)) at $p < 0.05$; * – $p < 0.01$.

polymorphic, but later, took on a rounded shape. When viewed under a microscope, preparations of live bacterial cells were noticeable for their high motility.

Examination of the physiological and biochemical properties of the isolates establishes that the isolated bacteria belong to aerobes, do not grow on meat peptone media and other protein substrates of animal origin, and their growth on nitrogen-free Ashby medium was also weak. The bacterial strains scantily utilize sugars, do not liquefy gelatin, and do not decompose starch, recover nitrates, or give a positive catalase test. According to the cultural, morphological, physiological, and biochemical properties, the bacteria classification fall into two genera, i.e., *Bradyrhizobium* and *Rhizobium.*

The study examined the PGP activity in the five most salt-tolerant isolates, including air nitrogen fixation and the production of the ACC-deaminase enzyme and the phytohormone IAA (Table 3). The rhizobia strains were able to capture air nitrogen, synthesize IAA, and have ACC-deaminase activity. On average, the three rhizobia strains, RH-6, RH-7, and RH-8, performed best. The main attribute in selecting salt-tolerant rhizobia was their ability to form nodules on soybean roots in saline soil conditions. Experiments with different salt concentrations ensued to investigate the nodulation ability of the different bacterial strains. Three rhizobia strains, RH-6, RH-7, and RH-8, served as inoculants. After 45 days of cultivation, nodules counting followed, with their dry weight and roots determined, along with nodule nitrogen fixation under salt stress conditions (Table 4).

Despite high salt stress, rhizobia created nodules on soybean roots in all the variants (Table 4). The number of nodules in the variants with salt stress decreased compared with the plants grown at the control (with no salt stress + inoculation with

Table 4. Nodulation and nitrogenase activity of rhizobia (variety Eureka).

Rhizobia	Number of nodules per plant (pcs)	Dry weight of nodules (mg/plant)	Root dry weight (g/plant)	Nitrogenase activity (μmole C_2H_4/g nodules/h)
No NaCl				
Control	0	0	10.3±0.1	0.00 ± 0.00
RH-6	44.2±2.0*	253.2±3.1*	36.0±0.3	27.71±0.2**
RH-7	48.4±2.1*	262.5±3.2*	39.8±0.3	29.89±0.3**
RH-8	46.1±2.2*	257.4±2.1*	38.7±0.2	28.67±0.1**
NaCl concentration @ 250 mM				
Control	0	0	7.6±0.1	0.00 ± 0.00
RH-6	28.5±1.3*	153.2±2.1*	23.6±0.3	25.59±0.1**
RH-7	32.5±1.1*	158.5±1.9*	26.8±0.3	27.75±0.3**
RH-8	29.3±1.4*	157.4±1.8*	25.7±0.2	26.34±0.2**
NaCl Concentration @ 500 mM				
Control	0	0	4.8±0.1	0.00 ± 0.00
RH-6	20.1±0.7*	136.1±1.2*	12.6±0.3	24.83±0.1**
RH-7	23.6±0.9*	141.6±1.3*	12.8±0.3	26.98±0.1**
RH-8	19.9±0.8*	138.1±1.1*	12.7±0.2	25.72±0.1**

Note: Control – without inoculation; Values are means ± standard error of the mean (SEM), n = 5; $p < 0.05$; ** and * indicate significance at the levels of 0.01 and 0.05, respectively.

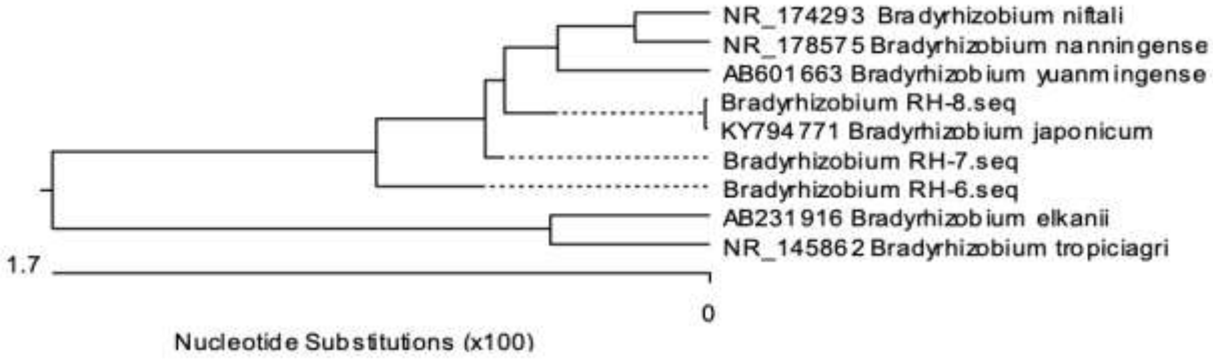

Figure 1. Neighbor-joining tree with bootstrapping 1000 replicates showing the phylogenetic relationship of three rhizobia and their most closely-related species based on the concatenated sequences from 16S rRNA.

rhizobia). Thus, at NaCl concentration of 250 mM, the number of nodules on the roots decreased by 33%–36%, and at 500 mM by 51%–56%. These variants also demonstrated the reduction in dry weight of nodules by 38%–39% at 250 mM NaCl and by 46% at 500 mM. Similarly, root dry weight decreased by 32%–34% at 250 mM and by 66%–67% at 500 mM, compared with the control variants (with no salt stress + inoculation with rhizobia). In these nodules, the nitrogen fixation also decreased slightly by 7%–10% compared with the control.

The bacteria authentification applied Sanger's molecular method based on the analysis of nucleotide sequences of 16SrRNA genes, entering the obtained nucleotide sequences of the strains into the BLAST program. The analysis revealed similarities with bacteria of the genus *Bradyrhizobium*. The phylogenetic analysis also took place by comparing the nucleotide sequence of strains with 16S rRNA sequences of related strains from the NCBI GenBank database and constructing phylogenetic trees using the MEGA 6.0 program with the Neighbor-Joining cluster method to formulate the genetic distances (Figure 1).

The phylogenetic analysis had strains RH-6, RH-7, and RH-8 assigned to the genus *Bradyrhizobium*. When identified in the Gene Bank, the strains occurred on the same phylogenetic branch as strain KY794771 *Bradyrhizobium japonicum S12*, which points to their belonging to this species and the degree of identity being 99.98% (Figure 1). Studying the effect of rhizobia inoculation on soybean salt tolerance on highly saline soil used the soybean cultivar Eureka, with three strains of salt-tolerant rhizobia, i.e., RH-6, RH-7, and RH-8 used for seed inoculation and seeds without (control). Data on the inoculation effect on nodule formation, number, and dry weight are in Table 5.

Soybean seed variants inoculated with salt-tolerant rhizobia significantly reduced the detrimental effects of salt stress. After two months of cultivation, nodules' number and dry weight were substantially ($P < 0.05$) higher in the rhizobia-inoculated variants compared with the control. Thus, the number of nodules enhanced by 2.3 ± 0.1 to 2.6 ± 0.2 times, and their nitrogen-fixing activity by 2.75 ± 0.3 to 2.9 ± 0.2 times compared with the control. The highest number of nodules (18.4 ± 1.1) and their nitrogen-fixing activity (6.81 ± 0.02) emerged by using the rhizobia strain RH-7 (Table 5).

The effect of salt-tolerant rhizobia inoculation on the growth and development of soybean plants in highly saline soils indicated that the inoculation boosts the dry weight of roots, shoots, and leaves in all the treated variants compared with the control (Figure 2). Injection of plants with salt-tolerant rhizobia significantly augmented the root's dry weight (by 1.65 ± 0.1–1.75 ± 0.1), shoot (by 2.0 ± 0.01–2.3 ± 0.2), and leaves (by 2.5 ± 0.2–3.5 ± 0.4) ($P < 0.05$) compared with the control. The highest dry weight of roots, shoots, and leaves showed in seed inoculation with rhizobia strain RH-7 (Figure 2). Inoculating with salt-tolerant rhizobia also significantly reduced the salt stress effects on soybean plants. Relevant differences between the variants with inoculation and the control also occurred. Inoculation leads to an enhancement in the root nodules, nitrogen fixation, plant growth, and stimulation of root development, which was the confirmation of the ecological adaptation of soybean plants to soil salinity.

Table 5. Effect of bacterial inoculation on the number of nodules, dry weight of nodules, nitrogenase activity (the Eureka variety).

Variants	Number of nodules per plant (pcs)	Dry weight of nodules * (mg/plant)	Nitrogenase activity ** (µmole C_2H_4/ml/h)
Control	7.1±0.2*	98.3±0.05*	2.32±0.01**
RH-6	16.2±1.3*	133.2±0.09*	6.41±0.01**
RH-7	18.4±1.1*	158.9±0.1*	6.81±0.02**
RH-8	15.1±1.4*	147.2±0.1*	6.39±0.01**

Note: Values are means ± standard error of the mean (SEM), n = 5; $p < 0.05$, ** and * indicate significance at the levels of 0.01 and 0.05, respectively.

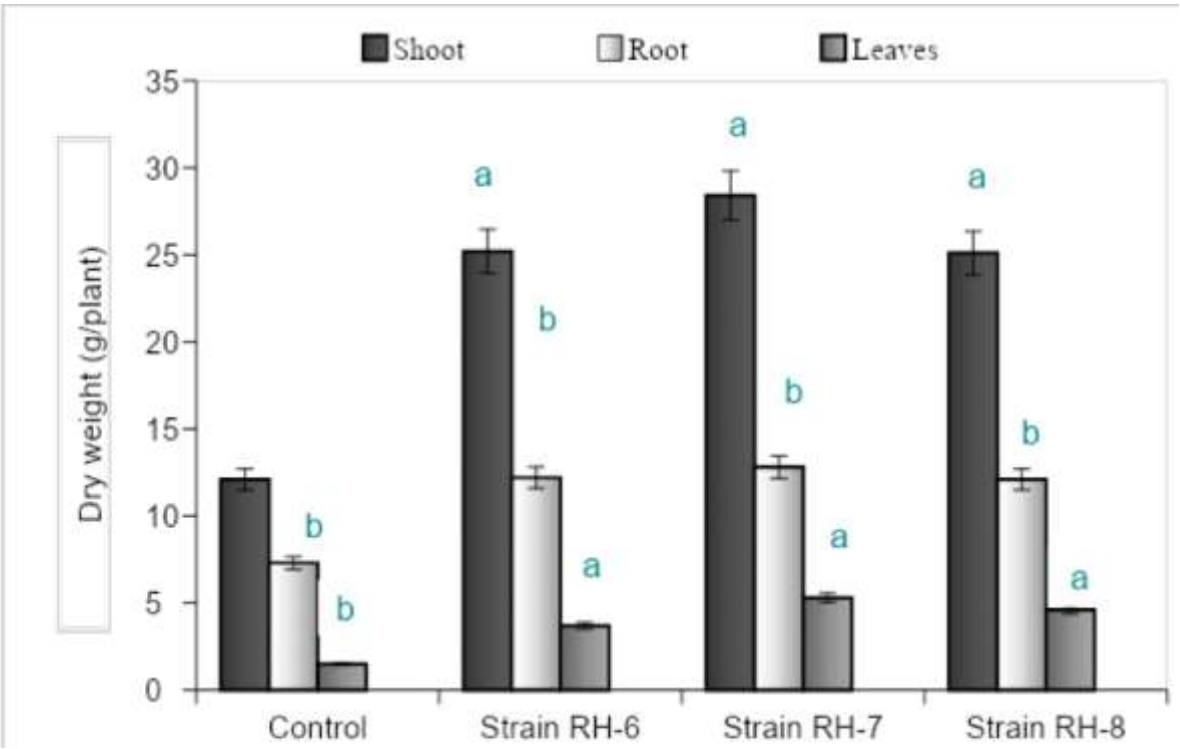

Figure 2. Effect of inoculation on the dry weight of shoots, roots, and leaves in highly saline soils. Control without inoculation, the Eureka soybean variety. Columns represent mean values while bars represent standard deviation (n = 5). Different letters show statistically significant different values ($p < 0.05$) between treatments as evaluated using the SNK-test.

DISCUSSION

In the presented study, rhizobia isolated from the root nodules of soybean grown on saline fields, underwent screening of isolates, with the highest salt tolerance selected. An important feature in the selection of salt-tolerant rhizobia was their ability to form nodules on the roots in saline soil conditions. The results further demonstrated that rhizobia form the nodules on soybean roots even at high salinity, and their nitrogen-fixing activity decreases insignificantly (by 7%–10%). The three most salt-tolerant strains selected and identified were RH-6, RH-7, and RH-8, as the variants of the species *Bradyrhizobium japonicum*.

Salt-tolerant microbes are known to help plants in reducing the salt stress effects through various mechanisms: stimulation of plant growth through the secretion of metabolites (Nagpal *et al*., 2020), induction of nutrient absorption (Albdaiwi *et al*., 2019), solubilization of phosphates, nitrogen fixation, and reduction in ethylene levels in plants (Shultana *et al*., 2020). The identified strains of salt-tolerant rhizobia produced the plant growth-promoting metabolites (phytohormone IAA and ACC deaminase) and gave the highest nitrogen-fixing capacity. The presence of IAA in rhizobia enhances the growth of soybean roots and nitrogen-fixing nodule formation, which eventually increases the nutrient uptake and helps the plants to cope with salt stress conditions. These assumptions were in analogy with the past studies as reported that IAA enhances the roots and shoots growth (Nawaz *et al*., 2020; Ali *et al*., 2022) and reduces yield losses due to salinization (El-Sabagh *et al*., 2022).

The enzyme ACC deaminase is another essential metabolite that helps crop plants adapt to salt stress conditions. With salt stress, the ethylene levels in plants increase, which negatively affects their growth. The enzyme reduces ethylene levels, prevents inhibition of negatively regulated genes involved in ethylene-induced plant stress, and activates the genes involved in plant growth (Orozco-Mosqueda *et al.*, 2020). Past studies demonstrated that the enzyme ACC deaminase synthesized by rhizobia inhibits the ethylene synthesis in soybean, and hydrolyzing ACC to ammonia and α-ketoburate supplies nitrogen and energy to soybean plants under salt stress conditions, which improves the plant's survivability and increases the soybean productivity on saline soils (Singh *et al*., 2022).

The study further revealed that the isolated salt-tolerant rhizome strains were very effective in promoting the growth of soybeans under saline soil conditions. It also confirmed that the root's dry weight increased by a factor of 1.65 ± 0.1–1.75 ± 0.1, the nodules' dry weight by a factor of 1.36 ± 0.1–1.61 ± 0.09, and the number of nodules by a factor of 2.3 ± 0.1–2.6 ± 0.2 compared with the control.

Existing facts explained that salt-tolerant rhizobia strains exhibited several functions that stimulate plant growth, such as generating phytohormone IAA and enzyme ACC deaminase; therefore, they could alleviate the salt stress effects on soybean plants. Thus, the conclusion that the presented study proves inoculation with salt-tolerant rhizobia reduces the detrimental effects of salinization on soybean plants. These results also align with past studies showing that salt-tolerant microorganisms mitigate the salt stress effects and enhance crop growth (Shultana *et al*., 2022).

We believe soybean inoculation with salt-tolerant rhizobia, the phytohormone IAA they produced enhances the soybean root's growth and the formation of nitrogen-fixing nodules, with the enzyme ACC deaminase suppressing the ethylene synthesis, which stimulates soybean growth and increases its ecological adaptation to the salinity of soils. These results are promising for use in the designated environmentally friendly rhizobia for growing soybeans on saline soils.

CONCLUSIONS

The role of stress-resistant strains of rhizobia with multi-faceted PGP properties in salt stress conditions is indispensable for solving problems related to crop production in saline soils. The salt-tolerant rhizobia can help soybean plants to cope with salt stress. Using salt-tolerant rhizobia will help restore the nutritional imbalances of plants by stimulating the growth of roots and increasing the number of nitrogen-fixing nodules. The ability of rhizobia to improve the plant's salt tolerance is an imperative criterion for agricultural applications. This study will benefit the sustainable development of food crops in regions prone to salinization Plans are in place to create biofertilizers to increase soybean productivity on saline soils based on active strains of rhizobia that can withstand salt stress. It is a very promising technology, as local strains of rhizobia can easily acclimate to natural conditions and synergize plant-microbial interactions to stimulate growth. Even though the study showed a high promise of inoculation of soybean seeds with salt-tolerant rhizobia to reduce the mudflow stress in plants, the mechanisms of this process have not been researched in depth and require further study.

FUNDING

The work has been completed in the framework of the grant project IRN AP09259080.

ACKNOWLEDGMENTS

This study used soybean (Glycine max (L.) Merr.) agronomic variety "Eureka". Seeds were kindly provided by Kazakh Research Institute of Agriculture and Plant growing LLC (department of fodder, oil crops, and corn). Almalybak village, Karasai district, Almaty region, Kazakhstan.

REFERENCES

Abduhu M, Khan AA, Mian IH, Mian MAK, Alam MZ (2018). Effect of seed treatment with sodium hypochlorite and hot water on seed-borne fungi and germination of okra seed. *Ann. Bangladesh Agric*. 22(2): 41–50.

Adsul AT, Chimote VP, Deshmukh MP, Thakare DS (2016). Genetic analysis of yield and its components in soybean (*Glycine max* (L.) Merrill). *SABRAO J. Breed. Genet*. 48(3): 247-257.

Alam T, Kurniasih B, Suryanto P, Basunanda P, Supriyanta, Ambarwati E, Widyawan MH, Handayani S, Taryono (2019). Stability analysis for soybean in agroforestry system with kayu putih. *SABRAO J. Breed. Genet*. 51(4): 405-418.

Albdaiwi RN, Khyami-Horani H, Ayad JY, Alananbeh KM, Al-Sayaydeh R (2019). Isolation and characterization of halotolerant plant growth promoting rhizobacteria from durum wheat (*Triticum turgidum* subsp. *durum*) cultivated in saline areas of the Dead Sea Region. *Front. Microbiol*. 10: 1639. https://doi.org/10.3389/fmicb.2019.01639.

Ali B, Wang X, Saleem MH, Sumaira, Hafeez A, Afridi MS, Khan S, Zaib-Un-Nisa, Ullah I, Amaral Júnior ATd, Alatawi A, Ali S (2022). PGPR-mediated salt tolerance in maize by modulating plant physiology, antioxidant defense, compatible solutes accumulation, and bio-surfactant-producing genes. *Plants* 11(3): 345. https://doi.org/10.3390/plants11030345.

Asad U, Asghari B, Naeem K (2021). Climate change and salinity effects on crops and chemical communication between plants and plant growth-promoting microorganisms under stress. *Front. Sustain. Food Syst.* 5: 618092. https://doi.org/10.3389/fsufs.2021.618092.

Chele KH, Tinte MM, Piater LA, Dubery IA, Tugizimana F (2021). Soil salinity, a serious environmental issue, and plant responses: A metabolomics perspective. *Metabolites* 11(11): 724. https://doi.org/10.3390/metabo11110724.

Das S, De TK (2018). Microbial assay of N2 fixation rate, a simple alternate for acetylene reduction assay. *Methods X* 5: 909-914. https://doi.org/10.1016/j.mex.2017.11.010.

Devkota KP, Devkota M, Rezaei M, Oosterbaan R (2022). Managing salinity for sustainable agricultural production in salt-affected soils of irrigated drylands. *Agric. Syst.* 198: 103390. https://doi.org/10.1016/j.agsy.2022.103390.

Didorenko SV (2021). Domestic selection of soybeans during the years of independence of Kazakhstan as the basis of the country's food security. Available at: https://kazniizr.kz/otechestvennaya-selektsiya-soi-v-gody-nezavisimosti-kazahstana-kak-osnova-prodovolstvennoj-bezopasnosti-strany/.

El-Sabagh A, Islam MS, Hossain A, Iqbal MA, Mubeen M, Waleed M, Reginato M, Battaglia M, Ahmed S, Rehman A, Arif M, Athar HUR, Ratnasekera D, Danish S, Raza MA, Rajendran K, Mushtaq M, Skalicky M, Brestic M, Soufan W, Fahad S, Pandey S, Kamran M, Datta R, Abdelhamid MT (2022). Phytohormones as growth regulators during abiotic stress tolerance in plants. *Front. Agron*. 4: 765068. https://doi.org/10.3389/fagro.2022.765068.

Guignard MS, Leitch AR, Acquisti C, Eizaguirre C, Elser JJ, Hessen DO, Jeyasingh PD, Neiman M, Richardson AE, Soltis PS, Soltis DE, Stevens CJ, Trimmer M, Weider LJ, Woodward G, Leitch IJ (2017) Impacts of nitrogen and phosphorus: From genomes to natural ecosystems and agriculture. *Front. Ecol. Evol*. 5: 70. https://doi.org/10.3389/fevo.2017.00070.

Haskett TL, Knights HE, Jorrin B, Mendes MD, Poole PS (2021). A simple in situ assay to assess plant-associative bacterial nitrogenase activity. *Front. Microbiol*. 12: 690439. https://doi.org/10.3389/fmicb.2021.690439.

Hernández-León R, Rojas-Solís D, Contreras-Pérez MM, Orozco-Mosqueda MDC, Macías-Rodríguez LI, Reyes-de la Cruz H, Valencia-Cantero E, Santoyo G (2015). Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by Pseudomonas fluorescens strains. *Biol. Control* 81: 83-92. https://doi.org/10.1016/j.biocontrol.2014.11.011.

Hossain S (2019). Present scenario of global salt-affected soils, its management, and importance of salinity research. *Int. J. Biol. Sci.* 1: 1–13.

Ilangumaran G, Schwinghamer TD, Smith DL (2021). Rhizobacteria from root nodules of an indigenous legume enhance salinity stress tolerance in soybean. *Front. Sustain. Food Syst.* 4: 617978. https://doi.org/10.3389/fsufs.2020.617978.

Iorgansky AI, Esimbekov MB, Tymakbaeva SA, Rakhmetzhanova AA, Koptyleu MA (2017).

Provision of arable soils of the Iliysky Alatau with mobile elements of mineral nutrition and its optimization by the use of mineral fertilizers for the leading crops of the region (on the example of a test farm – Baiserke-Agro LLP of the Talgar district of the Almaty region). *Soil Sci. Agrochem*. 4: 27–37.

Kaushal M, Kaushal R (2015). Acetylene reductase activity and molecular characterization of plant growth promoting rhizobacteria to know efficacy in integrated nutrient management system. *Indian J. Biotechnol.* 14: 221-227.

Lestari EG, Yunita R, Anshori MF (2022). Selection and validation of M4 soybean mutant lines through multi-characters approach. *SABRAO J. Breed. Genet.* 54(5): 1077-1089. http://doi.org/10.54910/sabrao2022.54.5.10.

Minhas PS, Ramos TB, Ben-Gal A, Pereira LS (2020). Coping with salinity in irrigated agriculture: Crop evapotranspiration and water management issues. *Agric. Water Manag.* 227: 105832. https://doi.org/10.1016/j.agwat.2019.105832.

Mohanavelu A, Naganna SR, Al-Ansari N (2021). Irrigation induced salinity and sodicity hazards on soil and groundwater: An overview of its causes, impacts, and mitigation strategies. *Agriculture* 11(10): 983. https://doi.org/10.3390/agriculture11100983.

Nagpal S, Sharma P, Sirari A, Gupta RK (2020). Coordination of *Mesorhizobium* sp. and endophytic bacteria as an elicitor of biocontrol against *Fusarium* wilt in chickpea. *Eur. J. Plant. Pathol.* 158: 143–161. https://doi.org/10.1007/s10658-020-02062-1.

Nawaz A, Shahbaz M, Asadullah IA, Marghoob MU, Imtiaz M, Mubeen F (2020). The potential of salt-tolerant PGPR in growth and yield augmentation of wheat (*Triticum aestivum* L.) under saline conditions. *Front. Microbiol.* 11: 2019. https://doi.org/10.3389/fmicb.2020.02019.

NCBI (2020). National Center for Biotechnology Information (NCBI). Available: htpp://www.ncbi.nlm.nih.gov (accessed on December 25, 2021).

Orozco-Mosqueda MDC, Glick BR, Santoyo G (2020). ACC deaminase in plant growth-promoting bacteria (PGPB): An efficient mechanism to counter salt stress in crops. *Microbiol. Res*. 235: e126439. https://doi.org/10.1016/j.micres.2020.126439.

Otie V, Udo I, Shao Y, Itam MO, Okamoto H, An P, Eneji EA (2021). Salinity effects on morpho-physiological and yield traits of soybean (*Glycine max* L.) as mediated by foliar spray with brassinolide. *Plants* 10(3): 541. https://doi.org/10.3390/plants10030541.

Qin P, Wang T, Luo Y (2022). A review on plant-based proteins from soybean: Health benefits and soy product development. *J. Agric. Food Res*. 7: 100265. https://doi.org/10.1016/j.jafr.2021.100265.

Ren S, Lyle C, Jiang G-L, Penumala A (2016). Soybean salt tolerance 1 (GmST1) reduces ROS production and enhances ABA sensitivity, and abiotic stress tolerance in *Arabidopsis thaliana*. *Front. Plant Sci.* 7: 445. https://doi.org/10.3389/fpls.2016.00445.

Saradadevi GP, Das D, Mangrauthia SK, Mohapatra S, Chikkaputtaiah C, Roorkiwal M, Solanki M, Sundaram RM, Chirravuri NN, Sakhare AS, Kota S, Varshney RK, Mohannath G (2021). Genetic, epigenetic, genomic and microbial approaches to enhance salt tolerance of plants: A comprehensive review. *Biology* 10(12): 1255. https://doi.org/10.3390/biology10121255.

Shultana R, Zuan ATK, Naher UA, Islam AKMM, Rana MM, Rashid MH, Irin IJ, Islam SS, Rim AA, Hasan AK (2022). The PGPR mechanisms of salt stress adaptation and plant growth promotion. *Agronomy* 12(10): 2266. https://doi.org/10.3390/agronomy12102266.

Shultana R, Zuan ATK, Yusop MR, Saud HM (2020). Characterization of salt-tolerant plant growth-promoting rhizobacteria and the effect on growth and yield of saline-affected rice. *PLoS One* 15(9): e0238537. https://doi.org/10.1371/journal.pone.0238537.

Singh RP, Pandey DM, Jha PN, Ma Y (2022). ACC deaminase-producing rhizobacterium Enterobacter cloacae ZNP-4 enhances abiotic stress tolerance in the wheat plant. *PLoS One* 17(5): e0267127. https://doi.org/10.1371/journal.pone.0267127.

Smirnova IE, Baimakhanova GB, Faizulina ER, Daugalieva ST, Tatarkina LG (2022). Isolation and identification of rhizobia promising for the creation of biofertilizer for soybean culture (*Glycine max* [L.] Merr.). *Sci. Rev. Biol. Sci.* 1: 38–43.

United Nations (2022). Take action for the sustainable development goals. Available:

https://www.un.org/sustainabledevelopment/sustainable-development-goals/.

Voss-Fels KP, Stahl A, Hickey LT (2019). Q&A: Modern crop breeding for future food security. *BMC Biol.* 17: 18. https://doi.org/10.1186/s12915-019-0638-4.

Wijewardana C, Reddy KR, Bellaloui N (2019). Soybean seed physiology, quality, and chemical composition under soil moisture stress. *Food Chem*. 278: 92–100. https://doi.org/10.1016/j.foodchem.2018.11.035.

Winand R, Bogaerts B, Hoffman S, Lefevre L, Delvoye M, Van Braekel J, Fu Q, Roosens NH, De Keersmaecker SC, Vanneste K (2020). Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: Comparative evaluation of second (Illumina) and third (Oxford Nanopore Technologies) generation sequencing technologies. *Int. J. Mol. Sci.* 21(1): 298. https://doi.org/10.3390/ijms21010298.

Yang MH, Jahufer MZZ, He J, Dong R, Hofmann R, Siddique KHM, Li FM (2020). Effect of traditional soybean breeding on water use strategy in arid and semi-arid areas. *Eur. J. Agron*. 120: 126128. https://doi.org/10.1016/j.eja.2020.126128.

You X, Nasrullah, Wang D, Mei Y, Bi J, Liu S, Xu W, Wang NN. (2022). *N^7-SSPP* fusion gene improves salt stress tolerance in transgenic *Arabidopsis* and soybean through ROS scavenging. *Plant Cell Environ*. 45(9): 2794–2809. https://doi.org/10.1111/pce.14392.