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INOCULATION OF *ACINETOBACTER JOHNSONII* GY08 TO ENHANCE THE GROWTH OF FABA BEAN (*VICIA FABA* L.) UNDER DIFFERENT SALT CONCENTRATIONS

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Abstract: Abiotic stresses affect microbial populations and soil chemical and physical properties resulting in significant yield losses of several crops. An important environmental component that impacts plant growth and development from seed germination to maturity is salinity. The objective of this study was to examine the effect of inoculating salt-tolerant rhizobacteria on the morphological and physiological characteristics of faba bean under different salt concentrations in pot experiments. Eight rhizobacterial isolates were tested for their salt tolerance ability on nutrient agar. One best tolerant isolate with the best tolerance, which showed better growth at higher salinity, was selected and evaluated for its effect on the faba bean. The experiment comprised six treatments with three replications in a completely randomized design, and the data was analyzed using a one-way analysis of variance. The results showed that seed germination decreased by 4.16% and 8.33% at 150mM and 300mM salinity, respectively. However, the application of *Acinetobacter johnsonii* GY08 significantly enhanced seed germination by 4.16% and 6.38% with 150mM and 300mM salinity, respectively, compared to the uninnoculated treatments with the same salt concentration. Plants inoculated with *Acinetobacter johnsonii* GY08 showed higher biomass, shoot, and root elongation than the uninnoculated plants under both non-saline and saline conditions. The findings indicated that *Acinetobacter johnsonii* GY08 facilitated the growth of faba bean seedlings under salinity stress conditions and enabled them to thrive by accumulating more proline compared to uninnoculated plants. Therefore, further studies on various varieties and under field conditions are recommended.

Key words: salt tolerance, germination, rhizobacteria, pgpr.

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Introduction

Both genetic and environmental factors affect plant characteristics and their adaptation to different environments. Salinity is one of the environmental factors that influence plant growth and metabolism from seed germination to maturity (Kamran et al., 2020). The quantity of dissolved salts in the soil solution is known as soil salinity. It can be caused by either natural or artificial sources (Stavi et al., 2021). Salinity has the potential to reduce global food production by up to 30% (Machado and Serralheiro, 2017). Soil salinization affects an estimated one billion hectares worldwide, with an annual increase of two million hectares (Nasrallah et al., 2022).

Based on data from 118 countries covering 73% of the global land area, more than 4.4% of topsoil and more than 8.7% of subsoil are salt-affected (FAO, 2021a). It affects crop production in almost one-fourth of the world's cultivable land with about 1.5 million ha falling out of production each year (FAO, 2021b). It is estimated that about 5% of the land in Africa (Rome, 2015), and 19 million ha of land in sub-Saharan Africa are affected by salinity (Tully et al., 2015). Ethiopia has the largest area of salt-affected soils in Africa, due to both human activities and natural sources. The land affected by salinity in the country amounts to 11,033,000 hectares (Fikadu and Jemal, 2022).

High salinity in soil or irrigated water is a huge threat to yields since it negatively affects the morphology, physiology, and yield of crops via osmotic stress and ion toxicity. It causes a variety of physiological impairments in plants. For example, the decreased stomatal conductivity limits the C-fixation capacity, disrupting the catalytic activities of the enzymes that fix carbon and destroy the photosynthetic pigments (Ullah et al*.,* 2021).

The faba bean (*Vicia faba* L.) is a widely cultivated crop in the highlands of Ethiopia, grown at elevations between 1800 and 3000 meters above sea level for multiple purposes (Misgana, 2017). In Ethiopia, faba beans cover about half of a million hectares of land, producing a total of 1.04 million tons and with a productivity rate of 2.1 tons/ha (Dereje and Debela, 2022). Despite its various benefits and the availability of high-yielding varieties (>3 t/ha) (MoALR, 2017), the national average yield of faba bean (2.11 t/ha) in Ethiopia has remained low compared to Egypt and the United Kingdom (3.47 and 3.83 t/ha, respectively) (CSA, 2018; FAOSTAT, 2018). This low productivity of the faba bean is caused by biotic and abiotic factors. Among the abiotic factors, soil salinity is a major limiting factor for faba bean production (Fekadu et al*.,* 2018). Since faba bean is moderately sensitive to salinity, it should be grown in soils with low or no saline content. Several studies have shown that saline conditions are harmful to faba bean genotypes (Abd El-Baki and Mostafa, 2014; Bimurzayev et al., 2021).

Plants have developed various ways to protect themselves from salinity stress, including morpho-physiological and molecular responses (Zhao et al., 2021). On the other hand, saline soils can be improved through leaching, chemical application, drainage systems, and the use of organic compounds such as sewage sludge, compost, and manure (Orhan, 2021). Another effective and eco-friendly method to improve saline soils is by using salt-tolerant plants (halophytes) and microorganisms. This approach does not require any additional chemicals or materials to be added to the already salt-affected soil.

To meet the global food demands, it is crucial to find ways to enhance soil health and create strategies that are tolerant to salinity stresses, and other constraining factors, while maintaining normal plant growth. One recommendation that has gained relevance in the agriculture sector is the use of microbial communities as bioinoculants (Elanahal et al., 2022). Hence, the hypothesis of this research was that plant growth promoting rhizobacteria (PGPR) could enhance the growth of faba bean in saline soils by the production of growth hormones, exopolysaccharides and solubilizing inorganic phosphates.

Previous research has demonstrated that the use of halotolerant or halophilic bacteria in areas affected by salt can enhance plant productivity and soil fertility (Grover et al., 2021; Habib et al., 2016). Therefore, this study aimed to investigate the effect of salt-tolerant rhizobacteria inoculation on the morphological and physiological characteristics of faba bean under varying NaCl concentrations in pot experiments.

Material and Methods

The study was conducted at the University of Gondar, Department of Biology microbiology laboratory (Gondar town, Amhara Region, Ethiopia). For this study, we employed the high yielding Dosha variety of faba beans acquired from the Gondar Agricultural Research Center, Gondar, Ethiopia. A total of eight rhizobacterial strains with multiple plant growth-promoting traits were obtained from the collection established in our previous research (Gebeyehu et al., 2024) as indicated in Table 1.

Table 1. Rhizobacteria strains and their accession numbers.

No.	Strain name	Accession number
1	*Acinetobacter* sp. GY01	OQ248105
2	Bacterial strain GY02	OQ248106
3	*Acinetobacter johnsonii* GY03	OQ248107
4	*Chryseobacterium* strain GY04	OQ248108
5	*Chryseobacterium proteolyticum* GY05	OQ248109
6	*Pseudomonas costantinii* GY06	OQ248110
7	*Pseudomonas chlororaphis* GY07	OQ248111
8	*Acinetobacter johnsonii* GY08	OQ248112

Salt tolerance test

The rhizobacteria strains were tested for salt stress tolerance on nutrient agar supplemented with 2, 4, 6, 8, 10, and 12% levels of NaCl (w/v) according to Albdaiwi et al. (2020). The result was designated as positive (+) and negative (-) for the presence and absence of growth, respectively.

Effects of *Acinetobacter johnsonii* GY08 on seed germination and vigor index under different NaCl concentrations

Healthy faba bean seeds of the same size were selected and then surface-sterilized with 70% ethanol for 1 minute. Afterwards, they were rinsed with 5% sodium hypochlorite for 3 minutes and washed with 3 changes of sterile distilled water. *Acinetobacter johnsonii* GY08 was grown in a volumetric flask containing 50ml of nutrient broth at 30°C in an incubator shaker at 120 rpm for 48 hours. The culture pellets were separated from the supernatant by centrifugation at 8,000 rpm for 10 minutes (Eppendorf 5425G Centrifuge). The pellet was then diluted in normal saline (0.85% w/v NaCl) to give a final concentration of OD600 = 1.00 (10^8 cfu/ml). Surface-disinfected bean seeds were soaked in the cell suspension for 1 hour (Bal et al., 2013). The experiment consisted of 6 treatments with 3 replications in a completely randomized design. The treatments were as follows: control (faba beans irrigated with tap water) (T0); faba beans irrigated with 150mM salt (T1); faba beans irrigated with 300mM salt (T2); faba beans treated with GY08 and irrigated with tap water (T3); faba beans treated with GY08 and irrigated with 150 mM salt (T4); and faba beans treated with GY08 and irrigated with 300 mM salt (T5).

A total of 10 broad bean seeds per treatment were placed in 9-cm Petri dishes with two layers of moistened filter paper. To ensure sufficient humidity for germination, 5mL of sterile distilled water (T0 and T3), 5mL of 150 mM NaCl solutions (T1 and T4), and 5mL of 300 mM NaCl solutions (T2 and T5) were added to the Petri dishes every 2 days. The seeds were incubated at 28°C in an incubator. Germination was considered to have occurred when the radicle had reached half the length of the seed. The germination rate was recorded for 7 days. After 7 days, the root and shoot lengths were measured, and the germination rate and vigor index were calculated according to ISTA (1996):

Germination rate (%) = number of germinated seed / total number of seed ×100, Vigor index = (mean of root length + mean of shoot length) × % of seed germination.

Pot experiment

The experiment comprised 6 treatments as described in the previous section. There were three replicates of each treatment and a completely randomized design was applied. Seeds were prepared as described in the section above. Faba bean seeds were planted in plastic pots with a diameter of 25cm and a depth of 30cm. The pots were filled with 3kg of sterilized agricultural soil and arranged randomly. To ensure proper growth, the plants were irrigated with non-saline water for the first 21 days. After 21 days, each pot received 200ml of salt solution (150 and 300 mM NaCl) every two days for 40 days. The plants were harvested after 60 days, and morphological (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight) and biochemical parameters (chlorophyll and proline contents) were determined. The roots and shoots of the faba bean plants were excised with a sterile blade and weighed separately on an electronic balance to compute the fresh weight. The dry weight of the root and shoot was measured after oven-drying at 70°C for 72 hours (Toghueo et al*.,* 2016).

To determine the chlorophyll content, the mature leaves of the treated faba bean were collected and one gram was measured for each treatment, then cut into fine pieces and crushed with a mortar and pestle. Following the method of Kamble et al. (2015), 5ml of 80% acetone was added and the mixture was carefully ground. After incubating for 3 hours at 4°C, the mixture was centrifuged at 8000rpm for 5 minutes (Eppendorf 5425G Centrifuge). The supernatant was transferred to a 50ml measuring cylinder, and the volume was increased to 20ml by adding 80% acetone. The absorbance of the solutions was measured at 663, 645, and 480nm using a spectrophotometer (Jenway 6405 Spectrophotometer), taking the 80% acetone solution as a blank. Readings were taken in triplicate samples and the average was used to calculate the chlorophyll content. The contents of chlorophyll a, b, and a + b (total chlorophyll) and carotenoids were calculated by applying the following formula (Arnon, 1949):

Chl.a= (12.7xA663)-(2.69xA645)/(1000xw)xv (mg/g),

Chl.b= (22.9xA645)-(4.68xA663)/(1000xw) x v (mg/ g),

Total chl. = (20.2 (A645) + 8.02 (A663)) / (1,001 x W) x v (mg/g), and

Carotenoids= (A480+0.114) (A663)-0.638 (A645)/ (1000xw) x v (mg/g),

where:

A = absorbance at a specific wavelength,

V = final volume of the chlorophyll extract in 80% acetone,

W = fresh weight of the tissue extracted.

The proline content was determined according to the method of Bates et al. (1973) using L-proline as a standard (Sigma-Aldrich). Firstly, 250 mg of leaves were homogenized in 5 ml of 3% 5-sulphosalicylic acid using a mortar and pestle.

Secondly, the homogenate was centrifuged at 8000 rpm for 20 minutes. Thirdly, the mixture of 1 ml of acid ninhydrin reagent, 1 ml of glacial acetic acid, and 1 ml of supernatant was incubated for 1 hour in a boiled water bath at 100°C, and the reaction was terminated by placing the mixture solution on ice for 10 minutes.

Finally, 2 ml of toluene was added to the mixture, and the absorbance of toluene was read at a wavelength of 520 nm using a visible spectrophotometer, with toluene serving as a blank. The serial concentrations of standard L-proline were 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml and their standard curve were made. Finally, the content of proline was determined from the standard curve of L-proline and its units were expressed as µmol g1 FW.

Statistical analysis

All data of faba bean growth parameters obtained from the plant growth promotion assay were analyzed by one-way ANOVA followed by the Tukey's test, with the bacterial inoculation method considered as an independent variable. All statistical analyses were calculated at the significance level $p \leq 0.05$ using SPSS software version 25.

Results and Discussion

Salt tolerance of the rhizobacterial strains

The results presented in Table 2 showed that the rhizobacterial strains exhibited a wide variation in their growth on the NA medium containing different concentrations of NaCl. Accordingly, all tested rhizobacterial isolates were tolerant to a concentration of 2–4% NaCl (w/v). Five strains were able to grow at a concentration of 6–10% NaCl. Only two rhizobacterial strains GY07 and GY08 survived at a salt concentration of 12% NaCl (w/v). *Acinetobacter johnsonii* GY08, which showed the best growth on nutrient agar supplemented with high NaCl content (12% w/v), was selected for this experiment. This isolate also showed multiple plant growth-promoting characteristics including phosphate solubilization, indole acetic acid (IAA), exopolysaccharide, and protease production. Salt-tolerant PGPR can help to alleviate soil salinity stress during plant growth, and bacterial exopolysaccharide (Astorga-Eló et al., 2021) can also help to alleviate salinity stress by lowering the amount of Na^+ available for plant uptake (Upadhyay et al., 2011).

Table 2. Salt tolerance of the rhizobacterial strains.

Isolate	NaCl concentrations (w/v)					
	2	4	6	8	10	12
Acinetobacter sp. *GY01*	+++	+++	++	+	+	-
Bacterial strain GY02	+++	++	++	+	-	-
Acinetobacter johnsonii GY03	+++	+++	+++	+	+	-
Chryseobacterium sp. strain GY04	+++	+++	+	+	-	-
Chryseobacterium proteolyticum GY05	+++	+++	+++	++	+	-
Pseudomonas costantinii GY06	+++	+++	+++	+	-	-
Pseudomonas chlororaphis GY07	+++	+++	+++	++	+	+
Acinetobacter johnsonii GY08	+++	+++	+++	+++	+++	++

Note: - = growth not detected, + = low growth, ++ = moderate growth, +++ = high growth.

Effects of *Acinetobacter johnsonii* GY08 on faba bean seed germination under different concentrations of NaCl

The result showed that exposure to salt adversely affected the germination of seeds and their vigor index values. However, the presence of *A. johnsonii* GY08 improved the germination and vigor index values of both salt-treated and untreated plants. The data in Table 3 indicated that seed germination decreased by 4.16% and 8.33% at salinity levels of 150mM and 300mM, respectively. On the other hand, inoculation with *A. johnsonii* GY08 significantly enhanced the germination percentage of seeds under both normal and salt-stressed conditions. The application of *A. johnsonii* GY08 increased seed germination by 4.16% and 6.38% at salinity levels of 150mM and 300mM, respectively, compared to the uninoculated treatments with the same salt concentration. These findings are consistent with previous research that indicates that PGPR can mitigate the negative effects of salinity on seed germination (Metwali et al., 2015; Ji et al., 2020). Seed germination and early seedling growth are the most salt-sensitive stages of plant growth under environmental stresses. This is because the seedling root is in direct contact with the soil and is affected by many soil changes, including salt stress. The use of PGPR promotes seed germination, which may be due to the ability of PGPR to synthesize hormones such as indole acetic acid, gibberellic acid, and cytokinins. These hormones regulate cell division and promote seed germination. The inoculation of *A. johnsonii* GY08 also improved the vigor index of both salt-treated and untreated seeds. The highest vigor index of 220.5±0.32 was recorded in T3, while the lowest vigor index was observed in T2 (63.36±0.12).

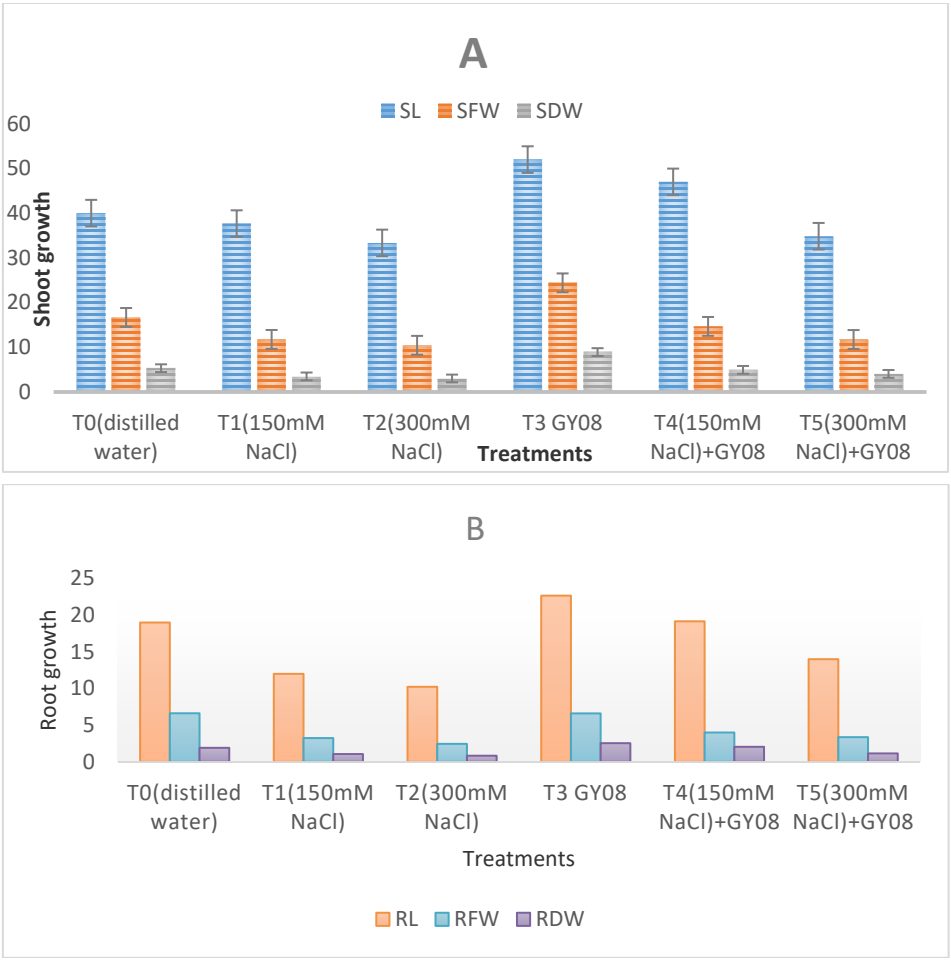
Table 3. Effects of *Acinetobacter johnsonii* GY08 on seed germination and vigor index in faba bean.

Treatment	Seed germination (%)	Vigor index
T0(distilled water)	96±0.01^{ab}	175.68±0.21^{b}
T1(150mM NaCl)	92±0.65^{c}	102±0.11^{c}
T2(300mM NaCl)	88±0.04^{d}	63.36±0.12^{d}
T3 GY08	98±0.5^{a}	220.5±0.32^{a}
T4(150mM NaCl)+GY08	96±0.32^{ab}	166.08±0.13^{ab}
T5(300mM NaCl)+GY08	94±0.21^{bc}	159±0.43^{bc}

Values are mean values ± standard deviation from three replicates. Different letters indicate a statistical difference between treatments (Turkey's test, $P < 0.05$) under normal and saline conditions.

Effects of *Acinetobacter johnsonii* GY08 on the growth parameters of faba bean under different concentrations of NaCl

The effect of *A. johnsonii* GY08 inoculation on faba bean growth was studied in both saline and non-saline soils. A significant ($p<0.05$) increase in the growth of faba bean inoculated with *A. johnsonii* GY08compared to control beans was found under both non-saline and saline conditions (Figure 1, Supplementary Table 1). Plants inoculated with *A. johnsonii* GY08 showed higher biomass, shoot, and root elongation compared to uninoculated plants under both non-saline and saline conditions. The highest growth increase in all growth parameters was recorded in T3 (52±0.23cm), while the lowest growth increase in all growth parameters was observed in T2 (33.33±0.09cm) (Figure 1 and Supplementary Table 1). These findings align with those reported by Metwali et al. (2015), who found that faba bean plants treated with *P. fluorescens*, *B. subtilis*, and *P. putida* exhibited significant growth stimulation as reflected in length, shoot fresh weight, and leaf area. The inoculation with *A. johnsonii* GY08 increased growth parameters in the presence of salinity stress and this may be attributed to the ability of PGPR to limit Na^+ and Cl^- transport into the shoots (Hmaeid et al., 2019). Similarly, Singh and Jha (2016) reported that inoculation of salt-tolerant PGPR under salt stress increased plant growth and yield. Thus, the PGPR strain *A. johnsonii* GY08 can be regarded as a promising microorganism for the formulation of biofertilizers especially suitable for saline soils to minimize faba bean crop yield reduction caused by soil salinization. *A. johnsonii* could be a potential plant growth promoting bacterium that produces indole-like compounds, exopolysaccharide and different hydrolysis enzymes and helps in nutrient solubilization and mobilization (Bhattacharya et al., 2024, Gebeyehu et al., 2024).



Note: SL – shoot length, SFW – shoot fresh weight, SDW – shoot dry weight, RL – root length, RFW – root fresh weight, RDW – root dry weight.

Figure1. The effect of *Acinetobacter johnsonii* GY08 on the morphological growth parameters of faba bean (A, shoot; B, root) under different NaCl concentrations.

Effects of the inoculation with *Acinetobacter johnsonii* GY08 on the photosynthetic pigments of faba bean plants under controlled and salt-stressed conditions

Plants exposed to salt stress exhibited a significant decrease in their photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoids. However, plants inoculated with *A. johnsonii* GY08 showed increased

photosynthetic pigments compared to the control plants under both saline and non-saline conditions (Figure 2). The plants treated with *A. johnsonii* GY08 and subjected to salt stress showed the highest chlorophyll contents, including chlorophyll a, chlorophyll b, and carotenoids, which were recorded at T3 (Figure 2 and Supplementary Table 2). This result is consistent with the findings of Yildirim et al. (2008), who reported that bacterial inoculants led to a significant increase in shoot/root dry weight, leaf number per plant, relative water content of the leaf, and chlorophyll content of the radish fruit.

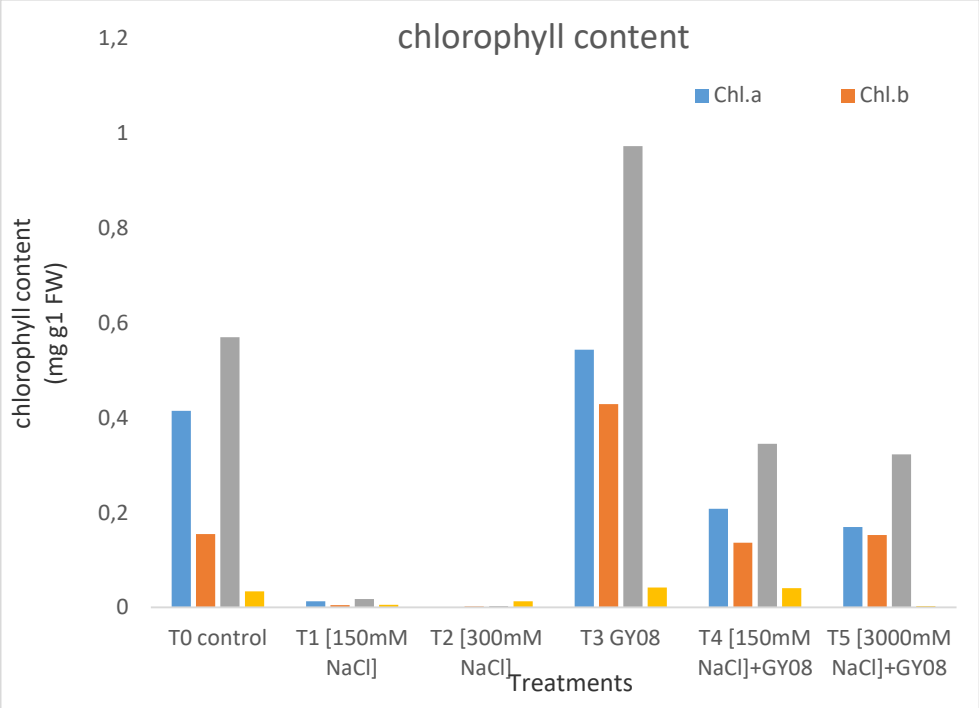


Figure 2. Effects of the inoculation with *Acinetobacter johnsonii* GY08 on the photosynthetic pigments of faba bean plants under controlled and salt-stressed conditions.

Effect of *Acinetobacter johnsonii* GY08 on osmolytes accumulation: proline content

The proline concentration (ug g1 FW) increased in the leaves of the salinity-stressed plants as compared to the control plants. In this context, T5 (300 mM NaCl +GY08) recorded the highest value of proline content. The minimum value was

recorded in the control pot treated with distilled water only. We observed that the accumulation of proline was increased in all inoculated and uninoculated plants under salinity stress conditions compared to the corresponding non-saline plants (Figure 3 and Supplementary Table 2). This supports the previous finding of Metwali et al. (2015) that rhizobacterial inoculation in faba bean increases the accumulation of proline. The inoculated plants had higher proline content than the uninoculated plants. The results of the current study suggest that proline content played an active role in defense responses and was upregulated by PGPRs. Salt stress causes a decrease in water uptake efficiency, leading to an imbalance in osmotic pressure and apoptosis. Proline acts as a compatible osmolyte, attempting to counteract the effects of salt stress (Albdaiwi et al., 2020).

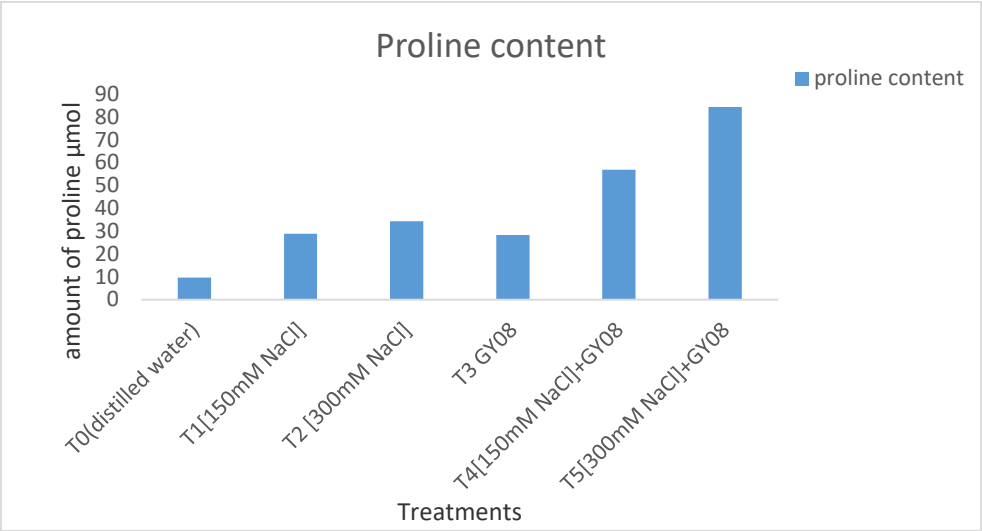


Figure 3. The effect of the inoculation with *Acinetobacter johnsonii* GY08 on the proline content of faba bean plants under controlled and salt-stressed conditions.

Conclusion

The results of the present study suggest that the rhizobacterial isolate *Acinetobacter johnsonii* GY08 facilitated the growth of faba bean seedlings under salinity stress conditions in the Dosha variety and allowed them to thrive. *Acinetobacter johnsonii* GY08 possesses a great potential to increase crop productivity and its association could reduce the negative impacts of salinity. Several field trials with different varieties under various field conditions are recommended for further utilization of this isolate as an option for salt stress management.

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POBOLJŠANJE RASTA BOBA (*VICIA FABA* L.) PRI RAZLIČITIM KONCENTRACIJAMA SOLI INOKULACIJOM SA *ACINETOBACTER JOHNSONII* GY08

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R e z i m e

Abiotski stresovi utiču na mikrobiološke populacije i hemijska i fizička svojstva zemljišta, što rezultira značajnim gubicima prinosa useva. Važna komponenta životne sredine koja utiče na rast i razvoj biljaka, od klijanja semena do zrelosti, je salinitet. Cilj ovog rada je bio da se ispita uticaj rizobakterija tolerantnih na povišen sadržaj soli na morfološke i fiziološke karakteristike boba pri različitim koncentracijama soli. Testirano je osam izolata rizobakterija kako bi se utvrdila njihova sposobnost tolerancije povišenog sadržaja soli u hranljivom agaru. Izolat koji je pokazao najbolji rast pri visokom salinitetu je odabran i procenjeno je njegovo dejstvo na rast boba. Eksperiment je obuhvatio šest tretmana sa tri ponavljanja u potpuno slučajnom dizajnu, a podaci su analizirani korišćenjem jednosmerne analize varijanse. Rezultati su pokazali da je klijanje semena smanjeno za 4,16% i 8,33% pri salinitetu od 150 mM odnosno 300 mM. Međutim, primena *Acinetobacter johnsonii* GI08 značajno je poboljšala klijanje semena za 4,16% odnosno 6,38% pri salinitetu od 150 mM odnosno 300 mM, u poređenju sa tretmanima koji nisu inokulisani, a imali su istu koncentraciju soli. Biljke inokulisane sa *Acinetobacter johnsonii* GI08 pokazale su veću biomasu, izduženje izdanaka i korena u poređenju sa biljkama koje nisu inokulisane u uslovima sa i bez povećane koncentracije soli. Rezultati su pokazali da je *Acinetobacter johnsonii* GI08 olakšao rast klijanaca boba u uslovima stresa saliniteta i omogućio im da napreduju akumulacijom većih količina prolina, u poređenju sa neinokulisanim biljkama. Stoga, se preporučuju dalja istraživanja na različitim sortama i u poljskim uslovima.

Ključne reči: tolerancija na so, klijanje, rizobakterije, PGPR.

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