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Effects of Rhizobacteria Inoculation on Germination and Physiology Status of Tomato under Salt Stress

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Abstract: The aim of this work is to explore the effect of inoculation with native rhizobacteria strains isolated from steppe ecosystems on enhancing salt tolerance of tomato (*Solanum lycopersicum* L.). For this purpose, various rhizobacteria strains have been purified, identified, and then applied on tomato seeds, *in vitro* then in pots to study their effects on the germination parameters and growth under salt stress. Eight genera of rhizobacteria strains were identified; they displayed pertinent plant growth promotion features such as phytohormones biosynthesis, fixation of atmospheric nitrogen, and phosphate solubilization. The obtained results demonstrated that salinity negatively affected seed germination and impaired plant growth. In response to salt stress, uninoculated plants accumulated a significant amount of osmoregulators (proline and glycine betaine), and recorded a decrease in their chlorophyll content, compared to inoculated plants, where the salinity tolerance has been much better with a high seedling growth as well as high chlorophyll and low osmolyte contents. Among the eight strains used in this study, three (*Agrobacterium*, *Citrobacter* and *Enterobacter*) demonstrated their effectiveness in enhancing salt tolerance of tomato plants. The use of native rhizobacteria strains as biofertilizer could be a sustainable approach to improve plant growth in salinity-impacted regions.

Key words: Bacterial inoculation, biofertilization, plant growth-promoting rhizobacteria, osmoregulators, salt stress, *Solanum lycopersicum* L.

The rhizosphere is the most complex microbial habitat on earth, integrating a complex density of plant roots, soil and a diverse microbial consortium of bacteria, archaea, viruses, and microeukaryotes (Ahkami *et al.,* 2017). Since the agricultural labor was known, human has appropriated a large part of natural biodiversity in order to ensure and increase the yields of their crops. One of the methods known is using various groups of microorganisms, including beneficial free-living soil bacteria to improve the growth of crop plants through facilitating the resource acquisition and inhibiting the plant pathogen (Amaresan *et al.,* 2016). The interactions that exist in the rhizosphere between plants and microorganisms must become a major priority for farmers; in fact, the healthy growth of plants and the best way for taking up essential nutrients depends most on the perfect interaction of soil stability and their fertility (Ambrosini *et al.,* 2016). In the last few years researchers have intensified studies on plant-microorganism interaction in order to develop sustainable agriculture based on the contributions of soil microflora in improving crop yield. Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can increase the growth of the plant through diverse mechanisms (Ahemad and Kibret, 2014). PGPR can improve plant growth through the production of a variety of bioactive compounds such as phytohormones and several active enzymes (Khanna *et al.,* 2019) (Mülner *et al.,* 2020). PGPR can facilitate the nutrients uptake through phosphate mobilization, nitrogen fixation or iron acquisition, which enable plant's growth on nutrient-poor soils (Kumar *et al.,* 2019). They can also contribute to growth by sustaining the plant's health, they can protect plants through direct biocontrol via the production of harmful compounds for neighboring phytopathological microorganisms (Viaene *et al.,* 2016). Therefore, PGPR are of great value for plant productivity and health. Although much remains to be discovered about

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the potential of these bacteria, on improving crop production under stressful conditions. For this purpose, experiments were conducted to assess the effect of microbial inoculum isolated from native plant roots growing in Algerian steppe areas, on germination and growth physiology of tomato (*Solanum lycopersicum* L.) and their role in decreasing the salinity stress.

Materials and Methods

Source of rhizobacteria strains

The bacterial strains tested in this study were isolated from native plant roots growing in Algerian steppe areas (Fig. 1). The choice of area was based on the diversity of rhizospheric microorganisms that live in this area, adapted to extreme conditions (salinity and drought), and which can influence the tolerance of inoculated plants to similar stress conditions in other areas.

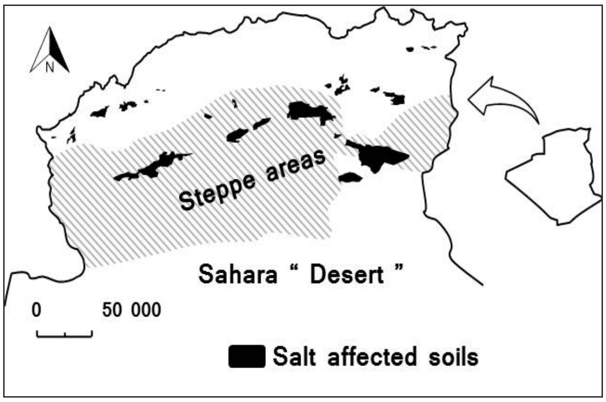

Fig. 1. Map of the steppe areas and salt soils in Algeria.

Isolation and strain purification procedures

The isolation of rhizobacteria was carried out according to the Vincent method (1972), and was done as follows: 10 g of rhizosphere soil was added to 90 mL sterile distilled water, then it was incubated on rotary shaker at 120 rpm for 10 min. 1 mL sample was serially diluted up to 10^{-7}, then 0.1 mL of diluted sample was plated on sterile nutrient agar medium and incubated for 3 days at 28°C. Single colonies were picked up and streaked on sterile nutrient agar medium plates to get pure culture.

Phenotypic and functional characterization of isolates

Well isolated colonies were observed for morphological characterization and, screened for their Gram's stain. Further identification was continued by standard biochemical and physiological tests supplemented as needed by plant growth promotion (PGP) activities tests such as inorganic phosphate solubilization (Kumar *et al.,* 2001), IAA production (MacWilliams and Maria 2009), fixation atmospheric nitrogen (Rodge *et al.,* 2016), and catalase enzyme production (Delarras 2007). Bergey's Manual of Determinative Bacteriology (David *et al.,* 2012) was used as a reference to identify the isolates.

Rhizobacterial strains evaluation on tomato seed germination under salt stress

Eight isolates (BC1…BC8) were selected to study their effect on the germination parameters (germination per cent and root length) under different levels of NaCl stress (electrical conductivity 2; 4 and 8 dS m^{-1}). The experimental approach of our work took place in several stages, which are summarized in the Fig. 2.

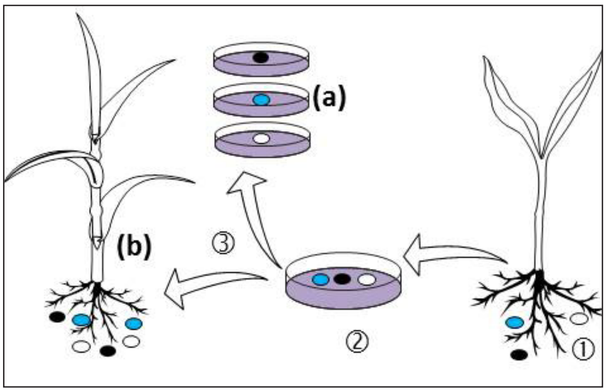

Fig. 2. Methodological steps of study. (1) Isolation of bacteria from native plants roots growing in steppe areas of Algeria, (2) Characterization and identification of strain isolates, (3) Evaluating of the effects of isolates on: (a) germination test in vitro (b) plant growth under salinity stress in the greenhouse.

The tomato seeds were surface sterilized with sodium hypochlorite 2% for 3 min and 75% ethanol for 3 min followed by repetitive washes with distilled water, and then they were soaked in each of the eight bacterial isolate suspensions separately (10^8 cfu. mL^{-1}) or in distilled water free from bacterial cells as control for 24 hours. A total of 12 seeds were sown in Petri dishes (9 mm diameter) on four sheets of Whatman number 1 filter papers. The seeds were moistened either with distilled water (control) or with solutions of varying NaCl concentration and incubated in growth

Table 1. Morphological and biochemical characterization of rhizospheric isolates

Characteristics	Code							
	1	2	3	4	5	6	7	8
Shape	Filamentous	Cocco-bacillus	Bacillus	Coccus	Cocco-bacillus	Cocco-bacillus	Coccus	Cocco-bacillus
Colony	Pale yellow	White	Orange	Pale yellow	Orange	Pink	White	Pale green
Gram Staining	+	-	-	-	-	-	-	-
Catalase	-	+	+	+	-	+	-	+
Fixation atmospheric nitrogen	-	-	+	+++	+	+	++	+
Indole production	-	-	++	+	+	+	+++	+
Solubilization of inorganic phosphate	-	+	+	-	-	+	-	+

1: BC1 *Streptomyces; 2:* BC2 *Acinetobacter; 3:* BC3 *Agrobacterium; 4:* BC4 *Azotobacter; 5:* BC5 *Flavobacterium; 6:* BC6 *Citrobacter; 7:* BC7 *Enterobacter; 8:* BC8 *Pseudomonas.*

room at temperature for 25°C in the dark, for 7 days, the emergence of the radicle from the seed was considered as an index of germination (Johnson and Wax, 1978). The germination rate was calculated as follows: Germination (%) = (number of seeds germinated/total number of seeds sown) × 100. The average root length of sprouted seeds is measured, by taking the total length of the roots for each treatment and dividing it by the total number of seeds (sprouted or not) (Darrah, 1993).

Rhizobacterial strains effects evaluation on tomato cultivars growth under salt stress

In order to remove all the microorganisms, the soil used was sterilized by autoclaving at 121°C for 30 min, before the inoculation of cultures, then divided into equal quantities (1.2 kg pot^{-1}). Some seeds were soaked in mix of the eight bacterial suspension or in distilled water (control) for 30 min, then they were sown in pots filled with sterilized soil (5 seeds pot^{-1} and 10 pot per treatment). The experiment was conducted in the green house at 25°C, relative humidity 60%, and plants were subjected to salinity stress by watering (electrical conductivity 2; 4 and 8 dS m^{-1}). Each experiment was replicated three times. Plant samples were collected and analyzed for chlorophyll, proline, and glycine betaine (GB) contents by following the methods described below: For the chlorophyll measurements, Chlorophyll Content Meter (model SPAD 502 Plus Konica Minolta) was used, measurements with the SPAD-502 meter produce relative SPAD meter values that are proportional to the amount of chlorophyll present in the leaf (Ling *et al.,* 2011). The method Paquin and Lechasseur (1979) was used for proline determination in leaves, the absorbance was measured at 520 nm with a UV-visible spectrophotometer. The GB content was determined by following the method used by Park *et al*. (2004), the absorbance was measured at 365 nm. The glycine betaine concentration is determined by referring to a standard curve prepared from a solution of the glycine betaine prepared in sulfuric acid on the basis of known concentrations.

Statistical analysis

Data were analyzed for significant mean differences via two-way Analysis of Variance (ANOVA) using XLSTAT software (version 2014). The effects of the bacterial inocula, compared with the negative control, were evaluated by multiple comparisons among class means using Dunnett's test.

Results and Discussion

Phenotypic and functional characterization of isolates

At the end of the microbiological analysis, eight (08) strains of rhizobacteria have been purified and identified in the genera: *Streptomyces*, *Acinetobacter*, *Agrobacterium*, *Azotobacter*, *Flavobacterium*, *Citrobacter*, *Enterobacter* and *Pseudomonas*, then they were coded respectively as: BC1, BC2, … BC8. Due to a large number of taxonomic species yet to be described, complete characterization has not

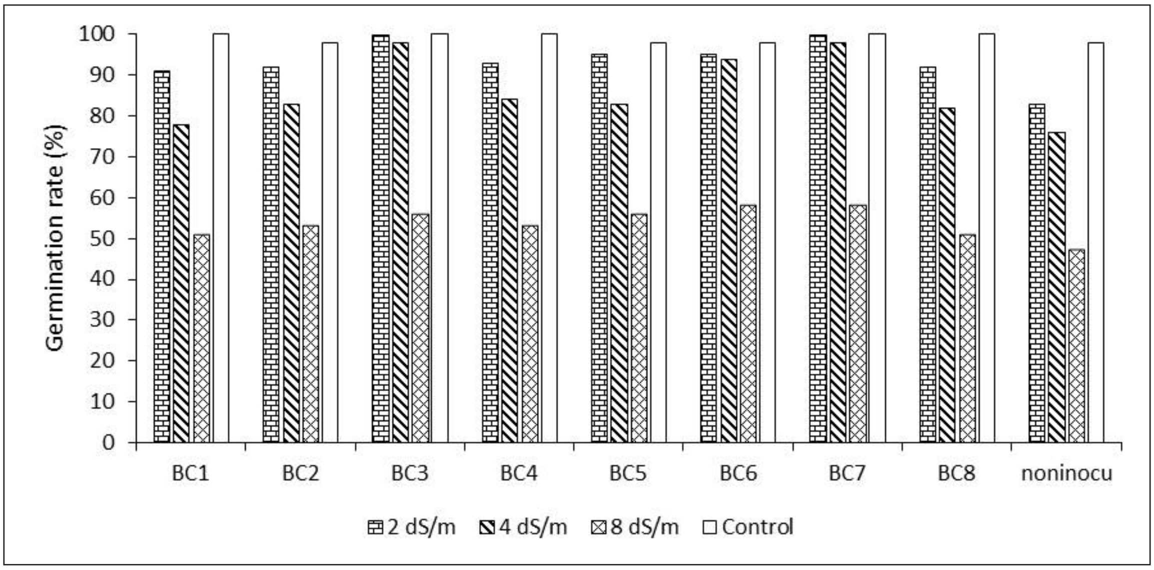

Fig. 3. Inoculation effects on seed germination percentage under salt stress.

been possible. All isolates were Gram-negative except BC1, inorganic phosphate solubilizing capacity was exhibited by BC2, BC3 and BC6 while the isolates BC4 had a good ability to fix atmospheric nitrogen. Out of the eight isolates, BC7 produced considerably higher amounts of IAA. Phenotypic and functional characterization of isolates were shown in Table 1.

Inoculation effects on seed germination under salinity stress

The results showed that under salt stress, most of the seeds undergo a reduction in their germination per cent compared to the unstressed seeds, except for inoculated seeds with BC3, BC6 and BC7 which were able to maintain a germination rate slightly close to 96% and exhibited significant difference at salinity levels of 2 and 4 dS m^{-1} compared to other strains. By applying the high salinity level (8 dS m^{-1}), germination percent did not exceed 60% for all strains (Fig. 3).

As with germination percent, root length was also significantly ($P \leq 0.05$) improved with inoculated seeds under salinity stress conditions. As may be seen below (Fig. 4), all of BC3, BC6 and BC7 strains were the most effective in enhancing germination percent and led to a considerable increase in root length.

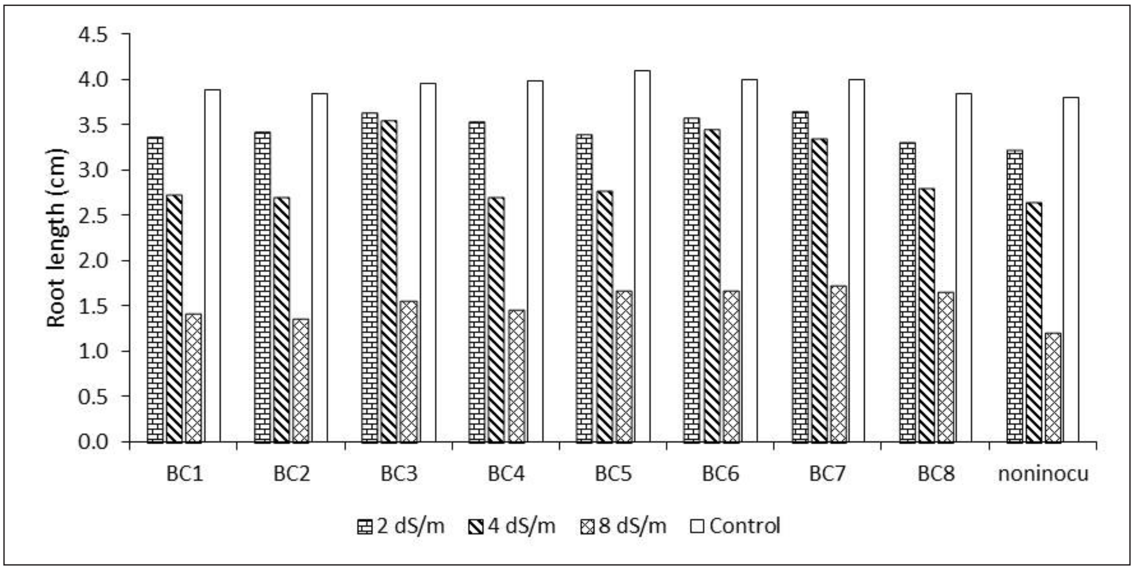

Fig. 4. Inoculation effects on root length under salt stress.

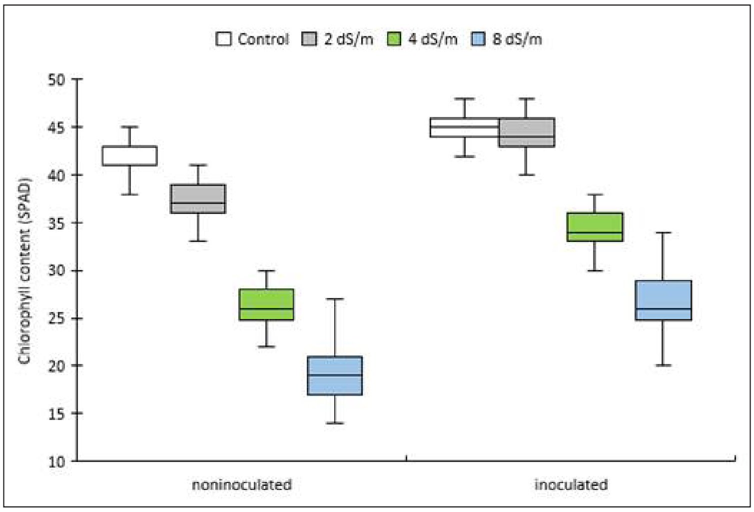

Fig. 5. Effect of inoculation on chlorophyll content under salt stress.

Inoculation effects on tomato seedling growth under salt stress

Determination of chlorophyll content: The data regarding chlorophyll content were collected after 60 days of culture, it showed that watering with salt water at different concentrations (2, 4 and 8 dS m^{-1}) induced a decrease of chlorophyll content in all treatments compared to unstressed control, this decrease was less in inoculated plants. Box-plots analysis (Fig. 5) revealed that the use of PGPR at different salinity levels was highly effective to enhance the chlorophyll content up to 44.13; 34.1 and 26.8 SPAD, respectively, for 2; 4 and 8 dS m^{-1} compared to uninoculated plants (37.13, 26.06 and 19.26 SPAD).

Determination of proline content: It was revealed from Fig. 6 that inoculation was very effective in decreasing the proline content under high salinity levels, the lowest proline content (46.83 and 50.91 µg mL^{-1}) were obtained with inoculated plants with a mix of all strains, these results differ significantly as compared to 51.52 and 55.25 µg mL^{-1} recorded with uninoculated plants treated respectively with 4 and 8 dS m^{-1} salt stress.

Determination of glycine betaine content: As shown in Fig. 7, under salinity stress the uninoculated plants accumulate a significant amount of glycine betaine, its content increased from 273.83 to 306.83 and then to 336.86 µg mL^{-1} respectively for the three varying NaCl concentration (2, 4 and 8 dS m^{-1}). However, this increase was less significant in the case

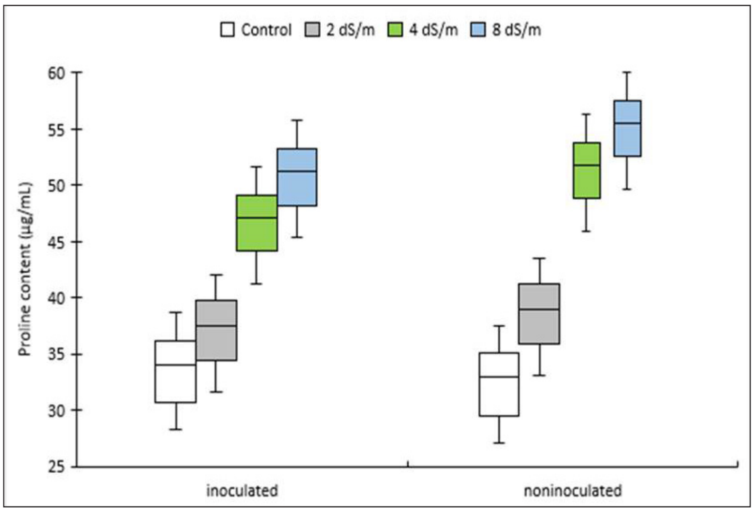

Fig. 6. Effect of inoculation on Proline content under salt stress.

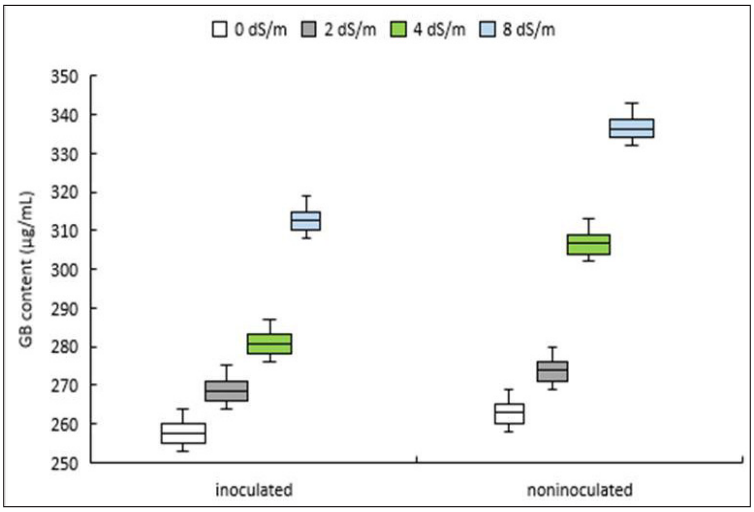

Fig. 7. Effect of inoculation on Glycine betaine content under salt stress.

of plants inoculated with a mix of all strains; 268.56, 280.56 and 312.86 µg mL^{-1} were recorded respectively for the three concentrations. Inoculation had considerable positive impacts on decrease of this osmolyte.

Discussion

The results of the present study revealed that germination parameters including germination per cent and root length were all significantly decreased with increasing salinity stress levels in uninoculated seeds, this trend has been reported by many researchers, Bano and Fatima (2009) on *Zea mays*, Kazachkova *et al.* (2018) on *Arabidopsis thaliana*, Singh *et al.* (2015) on wheat and Zhang and Blumwald (2001) on tomato. Previous studies attribute that to a decrease in the viability of seeds and slow or less mobilization of reserve foods (Cumming *et al.*, 2015) or suspending the absorption of water and nutrients (physiological drought) as suggested by Kazachkova *et al.* (2018). On the other hand, the same results indicate that the inoculated seeds are more resistant and their germination per cent higher than those uninoculated at the same salinity levels. In a general way, the effect of inoculation is manifested by an increase in germination per cent and root length. Several publications have appeared in recent years (Bhattacharyya and Jha, 2012; Egamberdieva *et al.*, 2019; Zerrouk *et al.*, 2020) documenting that there was a significant increase in germination per cent and root elongation after inoculation of seeds with some rhizobacteria strains. Wang *et al.* (2015) have proposed that the germination parameters can be used as selection criteria of rhizobacteria for their participation in seeds tolerance to salt stress. In this work and in related references it was observed that increased salinity reduced total chlorophyll levels but increased proline, and glycine betaine contents (Shabala *et al.*, 1998; Abiala *et al.*, 2018). According to Gharsallah *et al.* (2016) the decrease in chlorophyll content is due to the reorganization of the enzymatic functioning of salt stressed plants. In fact, glutamate, which is a precursor common to chlorophyll pigments and some osmolyte, is used more in the biosynthesis of osmolyte (Bresson, 2013). In this experiment, however, results showed that the proline and GB accumulation did not rise substantially in inoculated plants, which meant that they were less stressed. Several authors suggested that different PGPR including *Staphylococcus*, *Agrobacterium* and *Azotobacter* genus can improve the properties of soil and facilitate the uptake of essential nutrients (Barazani and Friedman, 1999) (Ipek *et al.*, 2014) (Viaene *et al.*, 2016). Also Esmaeil Zade *et al.* (2019); Ali *et al.* (2020), through their studies concluded that *Pseudomonas*, *Micrococcus* and *Enterobacter* genus can help plants to better tolerate environmental stresses by producing certain phytohormones, such as auxins (IAA), gibberellins and cytokinins. As well other PGPR, such as *Bacillus*, *Acinetobacter*, *Citrobacter* and *Flavobacterium*, have a good ability to produce a variety of bioactive compounds such as enzymes and bioactive peptides under drought, heavy metals, and salts stress which can contribute to alleviate the stress (Kumar *et al.*, 2019; Khanna *et al.*, 2019; Mülner *et al.*, 2020). Therefore, they are of high value for plant productivity and health. From this study, it is clearly demonstrated that the adverse effects of salinity could be partially or fully compensate by the rhizobacteria inoculation, it is possible to conclude that the Rhizobacteria and their interactions with plants hold great promise for enhancing the crops production and may be useful in agronomic situations where soil salinity is diagnosed as a problem.

Conclusion

The study of rhizospherical interactions (plant-microorganisms) in the last few years has shown the capital role of microorganisms in the harmony of plant growth and proves that they are sustainable partners. Consequently, farmers must know most of them, their mode of expression and the way to take advantage of them to enhance the crop production. The results of our study demonstrate clearly that salinity decrease the germination parameters, reduces total chlorophyll levels and increase osmoregulators contents. However, the inoculation with rhizobacteria strains isolated from Algerian steppe areas, promoted considerably tomato seedlings growth, raised chlorophyll content, and decreased the accumulation of osmoregulators contents (proline and glycine betaine). Summing up the above results, it clearly appears that rhizobacterias are considered as ecological and economical alternative to reduce the high salinity effect and can be used as an efficient biofertilizer in crop plants in salinity-impacted regions mainly in arid regions.

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