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Investigating the effects of plant growth-promoting rhizobacteria isolates on germination and physiology status of durum wheat under salt stress

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Abstract: The aim of this work is to evaluate the seedling growth and physiology status of wheat seeds inoculated with a suspension of eight plant growth-promoting rhizobacteria (PGPR) isolates. For this purpose, rhizobacteria strains were isolated from the roots of native plants growing in the Algerian steppe, then evaluated for their plant growth promotion (PGP) features, and finally applied on wheat seeds. The obtained results showed that the majority of the tested strains displayed pertinent PGP features. In *in vitro* experiments, results showed that salinity affected negatively seed germination and impaired plant growth while the inoculation with BC3, BC6 and BC7 strains induced a good germination rate and improved significantly the root length. In greenhouse experience, data demonstrated that non-inoculated plants accumulated a significant amount of osmoregulators (proline and glycine betaine), and recorded a decrease of their chlorophyll content, compared to inoculated plants, where the salinity tolerance of this latter has been much better with a high seedling growth as well as high chlorophyll and low osmolyte contents. The results may be a useful extension of our knowledge of the interaction between plant and PGPR, in view of their possible applications as a biofertilizer to improve plant growth in salinity-impacted regions.

Key words: bacterial inoculation; biofertilization; PGPR; plant-microbe interactions; omoregulators; salt stress; durum wheat

Preučevanje učinka izolatov rizobakterij, ki pospešujejo rast rastlin na kalitev in fiziološke parametre trde pšenice pod slanostnim stresom

Izvleček: Namen raziskave je bil ovrednotiti rast sejank in fiziološko stanje trde pšenice, katere semena so bila inokulirana s suspenzijo devetih izolatov rizobakterij, ki pospešujejo rast rastlin (PGP). V ta namen so bili izolati rizobakterij izolirani iz korenin samoniklih rastlin, ki rastejo v alžirski stepi. Kasneje je bil ovrednoten njihov učinek na pospeševanje rasti rastlin, nakar so bili uporabljeni za inokulacijo semen pšenice. Dobljeni rezultati so pokazali, da je imela večina preiskušenih sevov pomembne PGP lastnosti. V *in vitro* poskusih so rezultati pokazali, da je slanost negativno vplivala na kalitev semen in zavrla rast rastlin med tem, ko je inokulacija z BC3, BC6 in BC7 sevi povečala kalitev in značilno izboljšala dolžino korenin. Poskusi v rastlinjaku so pokazali, da so ne inokulirane rastline značilno povečale količino osmoregulatorjev (prolina in glicin betaina), zabeležen je bil upad vsebnosti klorofila v primerjavi z inokuliranimi rastlinami. Toleranca inokuliranih rastlin na slanost je bila kasneje mnogo večja, kar se je pokazalo v boljši rasti, v večji vsebnosti klorofila in zmanjšanju osmotikov. Rezultati bi lahko bili koristni za izboljšanje razumevanja interakcij med rastlinami in PGPR, tudi z vidika njihove uporabe kot biognojil za izboljšanje rasti rastlin na zasoljenih območjih.

Ključne besede: bakterijska inokulacija; biognojenje; PGPR; interkacije rastline-mikrobi; omoregulatorji; solni stres; trda pšenica

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1 INTRODUCTION

Soil is the main support for most agricultural products. It also represents a heterogeneous environment that allows the development of many microorganisms, which are in continuous interaction with other species, under conditions of symbiosis, antagonism, mutualism, parasitism and saprophytes (Gouda et al., 2018; Bhat et al., 2019). For a long time, soil microbes were seen exclusively as pathogens, recent studies on plants and soil microbiome interactions have made this negative view obsolete (Lopes et al., 2021). The rhizosphere fungal and bacterial communities can harbor beneficial organisms. These organisms have the ability to colonize plant roots providing benefits to their hosts, by increasing the growth of the plant through the production of a variety of bioactive compounds such as phytohormones and several active enzymes and facilitating the nutrient uptake which enables plant growth in nutrient-poor soils (Khanna et al., 2019; Mülner et al., 2020), they can also contribute to protecting plants through direct biocontrol via the production of harmful compounds for neighboring phytopathological microorganisms (Viaene et al., 2016). Cultural practices such as plowing cause a decrease in the diversity of soil-borne microorganisms, a phenomenon amplified by the introduction of xenobiotic compounds (pesticides, chemical fertilizers) and soil salinization due to climate change (Marlet & Job, 2006), in this situation, it is unlikely that cultures will have a chance to naturally recreate an optimal microbial ecosystem.

Algeria produces more than 60 % of its cereals on agricultural land that is situated in salinity-prone areas. However, salinity is one of the primary factors limiting yield in these regions (Djermoun, 2009). According to FAO, land salinization should be considered a major risk likely to affect around 25 % of irrigated areas or 10 % of global food production. (FAO, 2015). Technical measures exist to control this phenomenon, but their application can take a long time and its implementation can reach considerable costs. To remedy this problem, the establishment of sustainable and environmental-friendly systems such as biofertilization can be a solution. For this purpose, present study was conducted to test the hypothesis that the isolated rhizobacteria have multiple PGP traits and that they can be used as biofertilizers to promote wheat (*Triticum durum* L.) growth under salinity stress.

2 MATERIALS AND METHODS

2.1 ISOLATION AND STRAIN PURIFICATION PROCEDURES

The bacterial strains used in this study were isolated from the rhizosphere of the halophyte plant Sparte (*Lygeum spartum* L.), which is found in steppe regions of Algeria. Location of the soil samples collection site: 34°35'51.8"N; 1°18'51.0"E. According to the Vincent, (1972) procedure, 10 g of rhizosphere soil was added to 90 ml of sterile distilled water, and the mixture was then incubated on a rotary shaker at 120 rpm for 10 min. Following this, 1 ml of the sample was serially diluted up to a concentration of 10^{-7}, and 0.1 ml of the diluted sample was then plated on sterile nutrient agar medium (containing 0.5 % peptone, 0.3 % beef extract, 1.5 % agar, 0.5 % NaCl, pH is adjusted to neutral 6.8) and incubated at 28 °C for three days. In order to obtain pure culture, single colonies were eventually picked up and streaked on sterile nutrient agar medium plates.

2.2 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF ISOLATES

Colonies that had been carefully isolated had their morphology examined, and their Gram stain was checked. Standard biochemical and physiological tests were used to confirm the identification, and plant growth promotion (PGP) activity assays, such as inorganic phosphate solubilization, IAA production, fixation atmospheric nitrogen, and catalase enzyme production.

2.2.1 Catalase Test

The catalase was revealed by depositing of a bacterial colony on a clean glass slide, in the presence of H_2O_2. Positive reactions are evident by immediate effervescence (bubble formation) (Delarras, 2007), the catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2$ + Catalase → $2H_2O + O_2$).

2.2.2 Indole acetic acids (IAA) test

According to MacWilliams (2009), the bacte-

rial isolates were examined for their ability to produce indole acetic acid (IAA). They were cultivated at 28 °C for 48 hours, in a liquid medium called "Tryptone Soya Broth" (TSB), and then 5 drops of Kovac's reagent were added by pouring them directly into the tube. The development of a pink to red colour in the reagent layer above the centre denotes a positive indole test.

2.2.3 Fixation of atmospheric nitrogen

According to the protocol of Rodge et al. (2016), bacteria were grown at 28 °C for 48 hours, on solid medium free of nitrogen «Ashby» or «Burk›s N-free» medium without mannitol. Any growth in this medium reveals the bacteria's capacity to fix atmospheric nitrogen.

2.2.4 Test for inorganic phosphate solubilization

The isolates were checked for phosphate solubilizing ability on the solid Pikovskaya (PVK) agar medium amended with 2 % tricalcium phosphate (TCP) (Kumar et al., 2001). Formation of a clear halo zone around the growth after 5 days of incubation indicates phosphate solubilizing ability.

2.3 RHIZOBACTERIA STRAINS EVALUATION ON WHEAT SEED GERMINATION UNDER SALT STRESS

Eight isolates (BC1…BC8) were selected to study their effect on the germination parameters (germination rate and root length) under different salinity levels (80 and 160 mM) developed with NaCl in sterilized distilled water. Seeds of the local Algerian durum wheat variety "Mohamed Ben Bachir" were used in this study. The experimental approach of our work takes place in several stages, which are summarized in the Figure 1.

The seeds were surface repeatedly washed with distilled water after being disinfected with sodium hypochlorite 2 % for 3 min and 75 % ethanol for 3 min. They were then immersed separately for 24 hours in each of the eight bacterial isolate solutions (10^8 CFU ml^{-1}). After being soaked with various NaCl solution concentrations, the treated seeds were incubated in Petri dishes at 25 °C in the dark. Only non-inoculated, unstressed seeds were used in subsequent tests as a control. The treatments of the experiment included the salinity at two levels: 80, and 160 mM, and seed inoculation with eight bacterial strains and three replicates per treatment for each strain (8 inocula × 2 treatments × 3 replicates). After 7 days, the emergence of the radicle from the seed was considered an index of germination (Johnson & Wax, 1978). The germination rate was calculated as follows: Germination (%) = (number of seeds germinated / total number of seeds sown) × 100.

By dividing the total length of the roots for each treatment by the total number of seeds (sprouted or not) the average root length of sprouted seeds is calculated (Darrah, 1993).

2.4 EVALUATION OF RHIZOBACTERIA STRAINS EFFECTS ON WHEAT CULTIVARS GROWTH UNDER SALT STRESS

Before installing cultures, the soil was autoclaved at 121 °C for 30 min to sterilize it, then it was divided into equal portions (1.2 kg each pot). Prior to planting, sterilized seeds were given 30 minutes to soak in a mix-

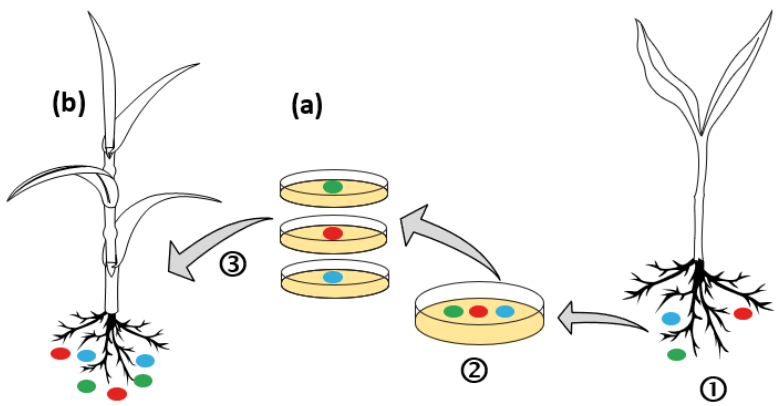

Figure 1: The scheme of the methodological steps of the study. 1: The isolation of bacteria from native plant roots growing in Algerian steppe areas, 2: The characterization and identification of strain isolates, 3: *in vitro* seed germination (a) and seedling development in greenhouse (b)

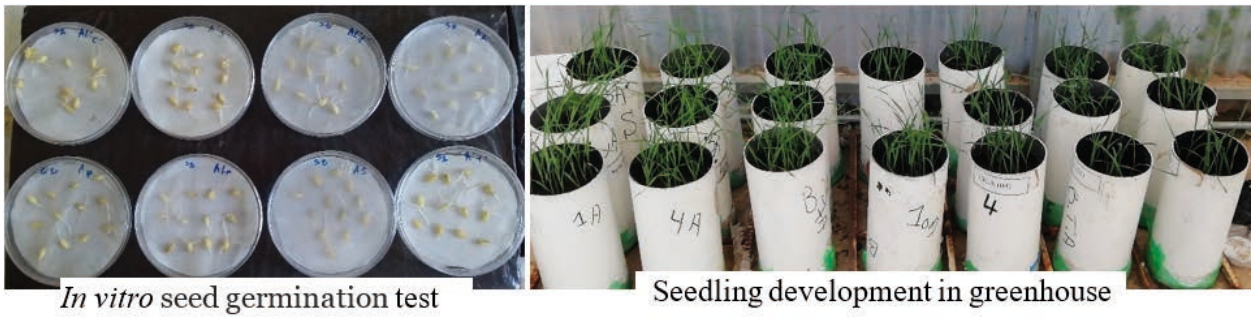

Figure 2: Effects of rhizobacterial strains on wheat cultivars growth under salt stress

ture of the eight bacterial suspensions. After that, the seeds were sowed in pots filled with sterilized soil and cultivated under salt stress conditions (80 and 160 mM) in the greenhouse at 25 °C, and 60 % relative humidity. Some sterilized seeds were sowed in pots without any salt treatment (unstressed control) (Fig. 2). Each treatment group comprised: 10 pots × 2 salt stress treatments × 3 replicates.

2.4.1 Leaf chlorophyll measurements

Plants stressed at 60 days after sowing were used for each salinity-restricted treatment, and the chlorophyll content of well-developed leaves was determined using a Chlorophyll Content Meter (SPAD 502 Plus). 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).

2.4.2 Determination of leaf proline content

Fresh leaves (100 mg) of each sample were chopped up and put in a test tube before being tested for proline using the Paquin & Lechasseur (1979) method. They were mixed thoroughlin 10 ml of 3 % sulfosalicylic acid aqueous solution ($C_7H_6O_6S$) and then filtered through Whatman filter paper. 2 ml of the filtrate was combined with 2 ml of ninhydrin ($C_9H_6O_4$) and 2 ml of acetic acid, glacial ($C_2H_4O_2$) in a 20 ml test tube. Samples were heated for 1 hour at 100 °C in a water bath. To stop the reaction, samples were put on ice, 4 ml of toluene was added to them. Then the whole mixture was vigorously stirred for 10 to 15 seconds. After standing for 20 min, a spectrophotometer was used to calculate the toluene portion's optical density at 520 nm. Finally, the standard range is established by pure proline.

2.4.3 Determination of leaf glycine betaine content

The amount of glycine betaine (GB) in each treatment was calculated using the technique described by Park et al. (2004). A sample of dry plant material (0.5 g) was combined with20 ml of distilled water. Sulfuric acid (NH_2SO_4) was used to dilute the resulting solution after it had been cultured for 48 hours at 25 °C. In cold water the resulting solution (0.5 ml) was chilled for 1 hour. After adding the reagent KI-I_2 (0.2 ml) the mixture was gently agitated using the vortex. Next, perform a 5-minute 14 000 rpm at 0 °C. After being aspirated, the supernatant is dissolved in 9 ml of 1,2-dichloroethane. First, it is washed and left with 0.5 ml for 5 minutes. After 2 hours, a UV-visible spectrophotometer was used to measure the absorbance at 365 nm. By using a standard curve created from a glycine betaine solution made in a sulfuric acid based on known concentrations, the glycine betaine concentration can be calculated.

2.5 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, were assessed using Dunnett's test for multiple comparisons among class means.

3 RESULTS AND DISCUSSION

3.1 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF ISOLATES

On the basis of phenotypic and functional characterization, the isolates were identified into 8 different strains, the latter were coded as: BC1, BC2, … BC8. Ex-

cept for BC1, all of the isolates were Gram-negative, BC2, BC3, and BC6 were able to dissolve inorganic phosphate while BC4 was effective at fixing atmospheric nitrogen. BC7 outperformed the other seven isolates in terms of production of IAA, The promotion of plant growth (PGP) features were found in the majority of isolates. Table 1 and Figure 3 provide a phenotypic and functional characterization of isolates.

3.2 INOCULATION EFFECTS ON SEED GERMINATION UNDER SALT STRESS *IN VITRO*

The findings show that there were differences in how durum wheat seedlings responded to salt stress based on salinity levels and inoculation treatments. Under salt stress, most of the seeds undergo a reduction in their germination rate compared to the unstressed seeds,

Table 1: Morphological and biochemical characterization of rhizospheric isolates

	Rhizospheric isolates							
Characteristics	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8
Form	Filamentous	Coccobacillus	Bacillus	Coccus	Coccobacillus	Coccobacillus	Coccus	Coccobacillus
Colour	Pale yellow	White	Orange	Pale yellow	Orange	Pink	White	Pale green
Gram Staining	+	-	-	-	-	-	-	-
Catalase test	-	+	+	+	-	+	-	+
Atmospheric nitrogen fixation	-	-	+	+++	+	+	++	+
Indole production	-	-	++	+	+	+	+++	+
Inorganic phosphate solubilization	-	+	+	-	-	+	-	+

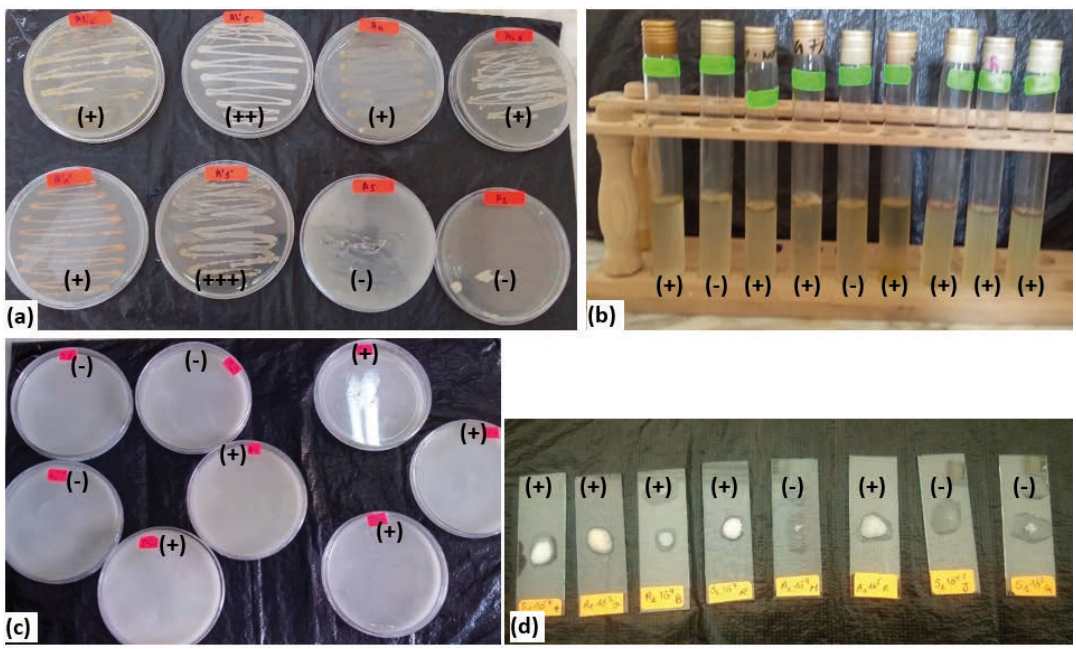

Figure 3: Screening of rhizospheric isolates for the plant growth promotion features. (a) Plates assay for screening of atmospheric N fixation, (b) Characterization of IAA producing isolates, (c) Plates assay for screening of inorganic phosphate solubilization, (d) Catalase test results, the absence of bubbling indicates a negative test. "+" indicates a positive test, whereas "-" indicates a negative test

however, the inoculated seeds were the exception, showing a much higher germination rate than non-inoculated. Among some isolates (BC3, BC6 and BC7) were able to keep their germination rates between 98 and 100 % slightly close to the witnesses (Fig. 4 A). Root length was also significantly improved with inoculated seeds under salt stress conditions. As may be seen below (Fig. 4 B), the highest root length was recorded with both BC3 and BC7 strains treated seeds.

According to the variables examined, the dendrogram providesgives the best depiction of the strains distribution into hierarchical clusters. The resulting tree's topology reveals two hierarchical clusters, one of which regrouped the three strains BC3, BC6 and BC7 that exercised a beneficial effect on the germination, seedling growth and give a clear capability to tolerate the salt stress as illustrated in Figure 5 A and B.

According to Johnson & Wax, (1978) the effect of inoculation is generally seen as an increase in germination rate and root length, two significant predictors frequently used to assess the success of the culture. Several publications have appeared in recent years (Egamberdieva et al., 2019; Rodge et al., 2016) documenting that, inoculating seeds with rhizobacteria strains significantly increases in germination rate and roots elongation. According to Egamberdieva et al. (2019) rhizobacteria might be chosen for their involvement in seed resistance to salt stress based on germination characteristics.

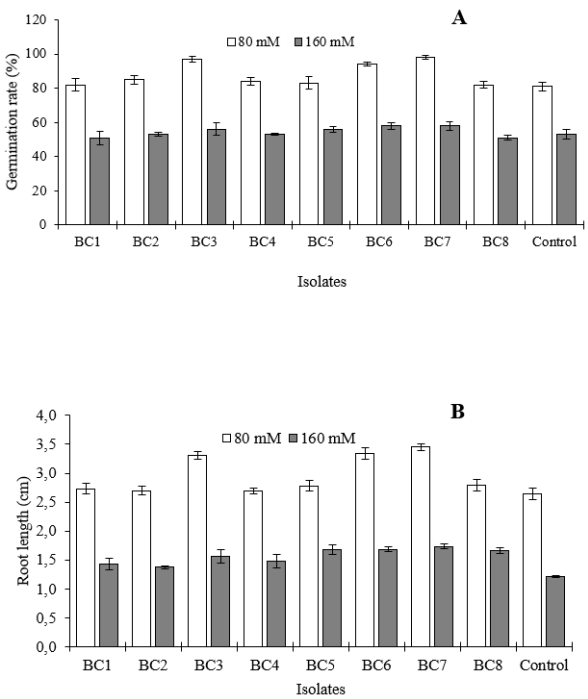

Figure 4: Inoculation effects on germination rates (A) and root length (B) under salt stress

3.3 INOCULATION EFFECTS ON WHEAT SEEDLING GROWTH UNDER SALT STRESS

3.3.1 Chlorophyll content

The results obtained for plant pigments after 60 days of stress showed that salinity leads to marked reductions in the total chlorophyll contents. In fact, the watering with the salt-water at different salinity levels induced a decrease of chlorophyll content in all treatments compared to unstressed control (Fig. 6). This reduction was less important in inoculated plants, where the total chlorophyll recorded was 28.31 and 21.13 significantly ($p < 0.05$) higher compared to 20.06 and 14.13 (in non-inoculated plants) respectively, for 80 and 160 mM.

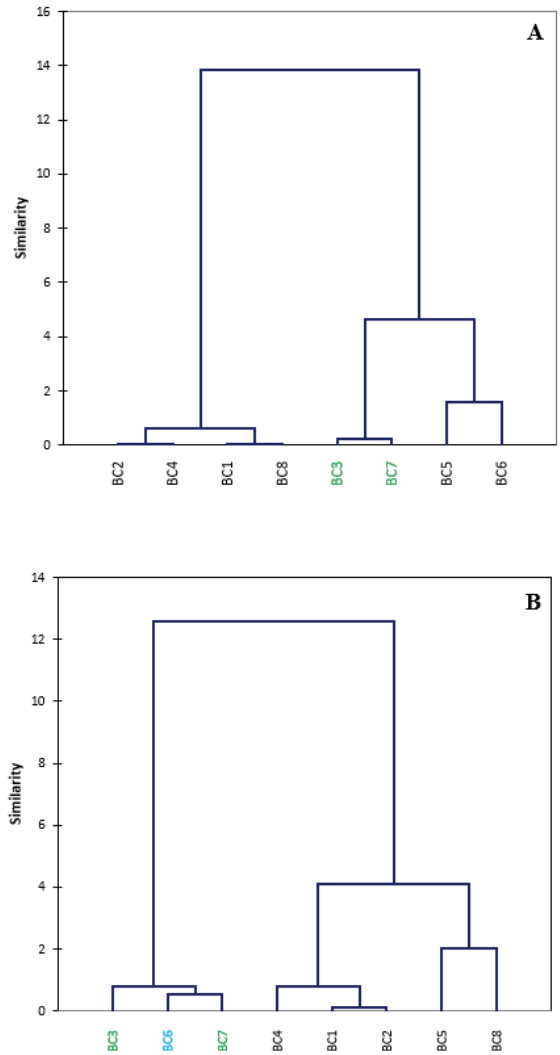

Figure 5: Dendrogram of the hierarchical ascending classification (ACH) of the inoculation effects according to the variables studied: **A**) germination rate. **B**) root length under salt stress

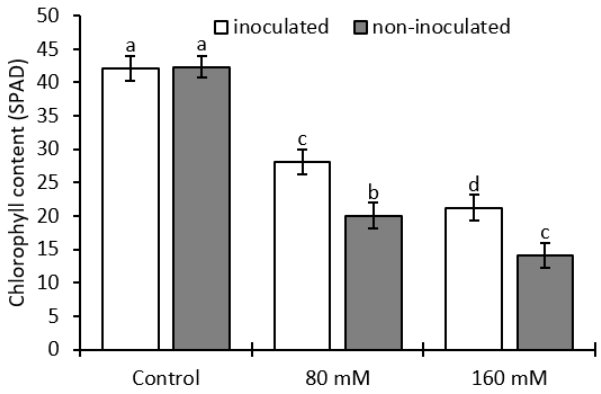

Figure 6: Effect of inoculation on chlorophyll content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

3.3.2 Proline content

The results obtained from pot experiments conducted in the greenhouse showe that the accumulation of proline varies according to the salinity levels and the inoculation treatment (Fig. 7).

Under different salinity levels, non-inoculated plants accumulate an important amount of proline (53.02 and 59.25 µg ml^{-1}), which is significantly different from the 48.28 and 54.91 µg ml^{-1} recorded in inoculated plants at 80 and 160 mM respectively. In this experiment, it was found that stress application resulted in non-inoculated plants losing chlorophyll content while simultaneously gaining proline content. Gharsallah et al. (2016) and Bresson, (2013) argue that this is due to the reorganization of the enzymatic function of the salt-treated plants, in fact, glutamate which is a common precursor of chlorophyll pigments and proline, is more commonly used in proline biosynthesis. According to Abiala et al. (2018), when plants are challenged by abiotic stress they frequently produce a stress response to try to mitigate the effects of the stressor and cellular solutes such as proline and sugars rise to confer desiccation tolerance.

3.3.3 Glycine betaine content

For all treatments, GB content increased with increasing salt concentration (Fig. 8), it increased from 327,83 to 361,75 µg ml^{-1} in non-inoculated plants, respectively for 80 and 160 mM. However, this increase was also significant for the inoculated plants (303,56 to 335,86 µg ml^{-1}).

Based on these findings , and the fact that salt stress in non-inoculated plants is also manifested by an increase in their GB content, it has recently been proposed by a number of authors (Amaresan et al,. 2016), that GB and proline are osmoregulators produced by a wide range of species, and are involved in stress resistance mechanisms. In fact, proline, soluble sugars, and GB accumulation, at the cellular level, during saline stress are primarily responsible for the maintenance of a high internal osmotic pressure (Chakraborty et al., 2012). This study revealed that when the salt stressed plants were inoculated with PGPR, the proline and GB accumulation did not significantly increase, indicating that they were less stressed. This may perhaps be due to a reduction in the growth inhibitory effect of salt on wheat plants through the enhanced activity of rhizospheric bacteria that can provide a variety of molecules which increases the tolerance of this plant.

Numerous microorganisms live in the rhizosphere of plants, and although for a long time, they were only thought of as diseases. This perception has been disproved by current research on the soil microbial popula-

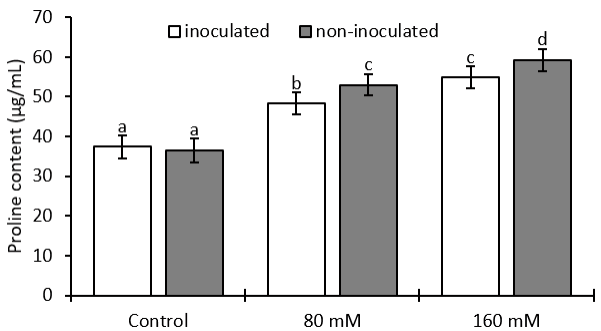

Figure 7: Effect of inoculation on proline content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

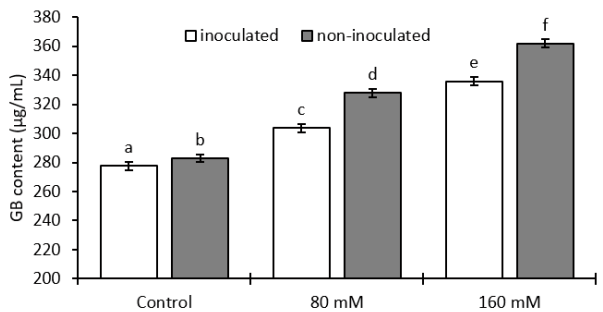

Figure 8: Effect of inoculation on glycine betaine content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

tion. Previous studies on the soil microbial community by Ambrosini et al., (2016) ; Bremer and Krämer, (2019) show that rhizospheric microorganisms are also capable of producing certain phytohormones andother similar compounds, which can enhance plant growth. Zerrouk et al. (2020); and Egamberdieva et al. (2019) have also found that the majority of rhizobacteria can boost plant tolerance by assisting with the uptake of specific nutrients, through symbiotic N_2 fixation, inorganic phosphate solubilization and organic phosphate mineralization.

4 CONCLUSIONS

Eight rhizobacteria's effects on the germination and the physiology status of wheat under salt stress were examined *in vitro* and in a greenhouse. Overall, the findings show that salt stress can be totally or partially offset by, inoculation with the tested rhizobacteria. We may deem the BC3, BC6, and BC7 strains as the most successful in reducing the negative effects of salt stress improvement in seed germination and a decrease in osmoregulators contents were seen, especially, after inoculation with BC3, BC6 and BC7 strains, so we can qualify them as the most effective to counteract the adverse effects of salt stress. From the research that has been carried out, we have demonstrated that inoculation with PGPR can modify the behavior of the plant and appeared as a biological solution to alleviate salt stress conditions in salinity-impacted regions mainly in arid regions.

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