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Potential of Halotolerant Rhizobacteria Isolated from Taif to Promote Growth and Alleviate the Salt Stress of Barley (*Hordeum vulgare* L.) Grown in Saline Soils.

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ABSTRACT

Forty three halotolerant bacteria, growing on nutrient agar medium supplemented with 10% NaCl were isolated from barley rhizosphere in Taif province. Based on *in vitro* tests, four isolates possessed activities of plant growth promoting rhizobacteria (PGPR) and two possessed higher activities. Based on morphological, biochemical and molecular characterization, they were identified. Phylogenetic analysis based on 16S rRNA analysis showed that all isolates belonged to *Bacillus* spp. For *in vivo* studies, pots were singly or dually inoculated with aqueous suspension ofthe most potent PGPR; *Bacillus subtilis* YHSA20 or/and *Bacillus* sp.YHSA34. Barley (*Hordeum vulgare* L.) seedlings were transplanted and 15 days old plants received 0, 100, 200, 300 and 400 mMNaCl, watered and received Hogland solution, each once a week and grown under greenhouse conditions for 12 weeks. Salt stress reduced the plant growth, leaf photosynthetic pigments, and caused considerable changes in proline, total phenolic compounds and peroxidase. Bacteria treated plants significantly improved photosynthetic pigments and plant growth, under salt stress. The bacteria ameliorated the salt stress, as indicated by increasing proline content and activities of peroxidase, in spite of lowering total phenolic compounds. This study indicates that these bacteria have potential to alleviate the salt stress and enhance plant growth under saline habitats, through direct and indirect mechanisms and could be appropriate bioinoculants under such conditions.

Keywords: halotolerant bacteria; salinity; salt stress; PGPR; *Hordeum vulgare*; *Bacillus subtilis*

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop worldwide and is an important source for feed, malt, and human food. It is a salt-tolerant crop species growing in arid and semiarid regions of the world. Salinity is one of the most harsh environmental factors limiting the productivity of crop plants. Abiotic stress leads to oxidative stress in the plant cell resulting in a higher leakage of electrons towards O_2 duringphotosynthetic and respiratory processes which leading toenhancement of reactive oxygen species (ROS) generation[1]. Naturally, many plants possess several mechanisms to decrease the negative effects of salinity including regulation and compartmentalizationof ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways [2,3]. Metabolic adaptation involves an increased synthesis of antioxidative compounds, such as proline, phenolic compounds and peroxidase enzyme enables plants to tolerate stress. Phenolic compounds play an important role in scavenging free radicals to protect plants against damaging with high levels of ROS [4]. Several strategies have been developed in order to decrease thetoxic effects caused by high salinity on plant growth, including plant genetic engineering [5], and the use of plant growth-promoting bacteria (PGPB) [6, 7,8]. Plant roots are colonized both by endocellular and intracellular microorganisms [9]. These rhizosphere microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments. They may enhance plant yield both directly and indirectly [7]. Microorganisms have many properties such as tolerance to saline conditions, genetic diversity, synthesis of compatible solutes, production of phytohormones, and their interaction with crop plants, that directly stimulate both plant growth and development [10]. Other microorganisms aid plants indirectly by protecting them against soil-borne diseases caused by some pathogenic fungi [11]. Certain microbes of PGPB like *Pseudomonas*, *Bacillus*, *Pantoea*, *Burkholderia* and *Rhizobium* are effective and provided tolerance against salinity in pea, maize, wheat, grapevine and common bean [12,13, 14,15,16]. *Bacillus* species like *B. subtilis* produced some metabolites in tomato plants, which stimulated the plant growth and alleviated the salt-ion toxicity [17]. The main aims of this work were to focus on the identification and characterization of halobacterial strains isolated from four different parts ofTaif province (Alhada; Ashayrah; Al Abar; Shogsan), and to evaluate the influence of selected bacteria on *Hordeum vulgare* growth and development under salt stress conditions.

MATERIALS AND METHODS

Collection of soil samples and isolation of bacterial strains

Soil samples were collected from barley rhizosphere at four different locations in Taif province, KSA including Alhada (h); Ashayrah (s); Al Abar (a); Shogsan (sh).Halotolerant bacteria were isolated from the selected soils.

Soil serial dilution was made from 10^{-1} to 10^{-6}. About 0.1 ml from each dilution was placed and spreaded on nutrient agar (NA) plates supplemented with 10% NaCl as a selective medium. Plates were incubated at 35 ºC for 30 days. Morphologically different microbial colonies were picked up. Mannitol Salt Agar (MSA) containing 7.5% NaCl[18] was used to confirm the salt tolerance ability of the isolates. Development of halo regions due to the fermentation of mannitol was a positive test for salt tolerance. Pure cultures were transferred to nutrient agarslants supplemented with 5 % NaCl and stored in refrigerator at 4 ^{o}C until used.

In vitro biochemical tests for the identification of PGPR strains

Biochemical tests viz, protease, chitinase, phosphate solubilization, indole acetic acid production, cellulose and starch hydrolysis were carried out for biochemical characterization of these isolates.

Phosphate solubilization

Phosphate solubilization by the isolates was checked in Pikovskaya's agar [19]. The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

Chitinase production

Chitinase hydrolysis activity was detected by the halo zones of clearance due to enzyme diffusion against the creamy background on the chitinase production medium around the bacterial colony. The medium contained the following componants (g/l); 1 colloidal chitin, 0.7 KH_2PO_4, 0.3 K_2HPO_4, 4 NaCl, 0.5 $MgSO_4{\cdot}7H_2O$, 0.001 $FeSO_4{\cdot}7H_2O$, 0.0001 $ZnSO_4{\cdot}7H_2O$, and 0.0001 $MnSO_4{\cdot}\ 7H_2O$ at 30ºC for 12days. [20].

Indole acetic acid (IAA) production

For detection of IAA, the selected bacterial cells were grown for 24 h to 48h in nutrient broth medium. Tryptophane (0.1 mM) was added in order to enhance IAA production by the bacteria [21]. Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere*et al*.[22]. For the reaction, 1ml of reagent, consisting of 12 g $FeCl_3$ per liter in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

Cellulase production

Cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar media with the following composition (g/l): KH_2PO_4 0.5, $MgSO_4$ 0.25, cellulose 2, agar 15, Congo-Red 0.2, gelatin 2; distilled water 1 L pH 6.8–7.2. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies [23].

Starch hydrolysis:

Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzymes, including α-amylase and oligo-1,6-glucosidase. It contains the following components (g/l): peptone 5, KCl 1, NH_4SO_4 1, $MgSO_4.7H_2O$ 0.5, NaH_2PO_4 1, soluble starch 30 and agar 20. pH was adjusted at 7.2 [24,25]. Iodine has been added to starch agar plate. The zone of clearing surrounding the isolatesindicate that they able to hydrolyze starch.

Protease production

Protease activity was detected on 3 % (w/v) powdered milk-agar plates [26]. 30 g of dried skim milk in 100 ml of distilled water was sterilized separately and was added to 900 ml of sterilized nutrient agar aseptically after cooling to 45-50 ºC. The zone of clearing surrounding theisolatesindicate that they produce the protease enzyme.

Selection and identification of the bacterial strains for *in vivo* plant growth promotion

Selection of the bacterial strains 20s and 34a was done based on the pervious *in vitro* tests for plant growth promotion activity. The selected isolates were subjected to many different morphological, biochemical and molecular characterizations.

Morphological characterization

Gram staining, motility and colony color were determined in 24 hour cultures in liquid nutrient medium supplemented with 5 % NaCl. Isolates were grown separately in Schaeffer's sporulation medium [27] for 24 h at 37 ºC. Spores were visualized by staining with malachite green and light microscopy.

Scanning electron microscopy (SEM)

Aliquots (5 ml) of freshly grown bacterial cultures (incubated for 24 h at 37 °C) were harvested, fixed, dehydrated and embedded essentially as described by [28]. Specimens were sputter coated with gold-palladium alloy and observed with a JEOL JSM-6390LA Scanning Electron Microscope (SEM) at 15 KV. (JEOL Electron Microscopy Ltd. Japan).

Biochemical characterization

Growth at different salt concentrations was determined by streaking each inoculum onto the surface of NA media with different concentrations of NaCl 0, 5, 10, 15, 20, 25 and 30% (w/v). Inoculated plates were incubated at 37 °C for 5 to 20 days. The temperature range was determined as above by incubating the bacteria at temperatures of 5, 15, 20, 25, 30, 40, 45, 50 and 55°C. The pH growth range was determined in a similar way on NA media adjusted at different pH values ranged from 5 to 11 and adjusted by HCl or NaOH. The ability to grow anaerobically was evaluated on solid medium incubated in jars with the GasPak envelops (BBL). Haemolysis was studied in solid Mueller Hinton (MH) medium supplemented with 5 % (v/v) defibrinated sheep blood. Oxidase reaction was performed according to Kovács[29]. Catalase was determined by adding 10 volumes of 3% H_2O_2 to each strain culture ontheir solid medium. Indole production was tested in liquid MH medium using Kovács' reagent [30]. Methyl red and Voges-Proskauer were tested using methyl red and Barritt's reagent [31], respectively. Starch agar was used to test the ability of an organism to produce certain exoenzymes, including a-amylase and oligo-1,6-glucosidase [24, 25]. Cultures were inoculated into tubes of nutrient gelatin (nutrient broth, 100 ml; Difco gelatin, 12 g; pH 7.0) and incubated at 37 °C for 14 days. After they had been cooled to 4 ºC, they were observed for liquefaction of the gelatin. Casein hydrolysis was indicated by a clear zone around bacterial growth on solid MH medium plus an equal quantity of skimmed milk [32]. Cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar media [23]. Chitin hydrolysis was measured by the halo diameter of enzyme diffusion on the chitinase production medium [20]. Production of acid from carbohydrates was determined by the methods of Gordon *etal*. [33]. Finally, Urea hydrolysis was detected on Christensen's medium [34].

Antimicrobial susceptibility tests

Antimicrobial susceptibility of the selected bacterial strains was assayed using the diffusion agar method [35]. The antimicrobial compounds (MASTRING-S^{TM}) used in this study included; Ampicillin (25µg) Tetracycline (30µg), Chloramphenicol (30µg), Levofloxacin (5µg), Flucloxacillin (5mcg), Tobramycin(10mcg), Ofloxacin (5mcg), Norfloxacin (10mcg), Cefotaxime (30µg), Imipenem (10µg), Bacitracin (10µg), Penicillin G(10µg), Polymyxin B(300U), Gentamicin(10µg) and Neomycin (30µg).

Molecular characterization

DNA extraction

Genomic DNA was prepared from overnight cultures of the selected strains grown separately in 1.0 ml of nutrient broth. Bacterial cells were washed thrice with 0.9 % NaCl and used to isolate total genomic DNA. DNA was isolated and purified by Macrogen extraction Kit following the manufacture protocol.

PCR experiments

Both the forward primer 5`-27F 5' (AGA GTT TGA TCM TGG CTC AG)3' and the reverse primer 5`-1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used for amplifying the complete 16S rDNA gene for the isolated DNAs of the strains. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µl reaction mixture by using a *EF-Taq* (SolGent, Korea) as follows: activation of Taq polymerase at 95 °C for 2 minutes, 35 cycles of 95 °C for 1minutes, 55 °C, and 72 °C for 1minutes each were performed, finishing with a 10- minute step at 72 °C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The sequencing primers were 785F 5'(GGA TTA GAT ACC CTG GTA)3' and 907R 5'(CCG TCA ATT CMT TTR AGT TT)3'. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

A fragment of 16S rDNA gene with a total length of 1350 bp has been sequenced for the strains. These data have been treated with Blastn program (http://www.ncbi.nlm.nih.gov) for searching for their closely related strain sequences already found in the Genbank database. Sequences with identity of 99 to 95 % to these two strains have been collected from the database. The collected data were used for phylogenetic analyses after unalignable and gap-containing sites were deleted (1350 bp in total). The aligned nucleotide

sequences can be obtained from the corresponding author upon request. The analyses were done by maximum-parsimony (MP) and neighbor-joining (NJ) by heuristic searches with the TBR branch swapping and 10 random taxon additions. Bootstrap replications were adjusted for both methods to be 10000 replications. The maximum-likelihood method in PAUP* 4.0b10 [36] was also used with NNI branch swapping and axis taxon additions and 200 bootstrap replications. The general reversible model (GTR+I+G) and parameters optimized by Modeltest 3.0 [37] were used.

Pot experiment

Grains of barley (*Hordeum vulgare* L. cv. Gustoe) were obtained from Tabuk Agriculture Company, KSA. They were surface sterilized with 0.2 % $HgCl_2$ solution for 5 min with frequent shaking and thoroughly washed with distilled water many times. Pot experiments were conducted in order to evaluate the effect of NaCl and bacterial inoculation on growth and salt tolerance of barley. They were performed in greenhouse in 3L pots in the Department of Biotechnology at Faculty of Sciences, Taif University, Taif, KSA. The experimental design was a full factorial design with five replicates per treatment. For inoculums preparation, both YHSA20 and YHSA34 strains were grown separately in nutrient broth (Merck, USA) for 24 h at 35 ºC; centrifuged at 3000 g for 20 min. The pellets were resuspended in distilled water, washed, rinsed twice, and finally resuspended to the same initial volume using 0.03 M $MgSO_4$. Twenty ml of the bacterial suspension ($3x10^8$ cells/ml) were used to inoculate each pot with the respect of bacterial strain. The bacterial suspension was pipetted on the surface of the pot and mixed thoroughly with the soil before barley seedling transplantation. Sets of non-inoculated pots were prepared as a control. All pots were irrigated weekly with different concentrations of sodium chloride (0, 100, 200, 300 and 400 mM), alternatively with water and 100 ml/pot nutrient Hogland solution, every three days until they were three months old. All pots were grown under greenhouse conditions at 25±2°C with 300 µmol $m^{-2}s^{-1}$PAR and a 16 h photoperiod.

Plant Growth analysis

Three months after sowing, barley plants were harvested to determine fresh and dry biomass. Dry biomass was determined after drying the plant in an oven at 70 ºC for three days.

Plant biochemical analysis

All biochemical analysis were performed from leaves of all sets of plants after three monthes of growth. Chlorophyll *a* and chlorophyll *b*contents were spectrophotometrically determined according to Lichtentaler and Wellburn[38]. One gram of barley leaves was extracted in 20 ml of 85 % aqueous acetone. The extract was centrifuged at 4000 *g* for 10 min. The supernatant was then taken and diluted by 85 % aqueous acetone to a 100ml. The absorbance was measured against a blank of pure 85% aqueous acetone at wavelengths of 645 and 662 nm. Both Chlorophyll *a* and *b* present in each samplewere calculated in µg g^{-1}FW according to Lichtentaler and Wellburn[38] using the following equations:

Chlorophyll a = 11.75 A662 – 2.350 A645
Chlorophyll b = 18.61 A645 – 3.960 A662

Determination of proline content

Proline content of barley leaves as a defense amino acid was determined according to Bates *et al*. [39]. A known dry weight (0.1 g) of leaves was extracted in 10 mL of aqueous 3 % sulfosalicylic acid over-night. The extract was centrifuged at 3000g for 10 min. Two mL of the supernatant was mixed with 2 mL of fresh acid ninhydrin solution and 2 mL of glacial acetic acid for reaction in a test tube for 1 h at 100 ºC. The reaction was terminated in an ice bath, and the mixture was extracted with 4 mL toluene. The extract was vigorously stirred for 20 s using a test tube stirrer. The chromophore-containing toluene was aspirated from the aqueous phase, and its absorbance was measured at 520 nm. Proline content was determined from a standard curve and calculated as mg g^{-1} DW.

Determination of antioxidative compounds

Total phenol content

The Folin-Ciocalteu method [40] was used to determine the total phenol content as antioxidative compounds. Gallic acid was used as a standard to produce the calibration curve. Total phenol content was expressed as µg of gallic acid g^{-1} DW.

Peroxidase enzyme

Half gram of fresh barely leaves were extracted in 50 mM sodium phosphate buffer (pH 7.0) and were assayed as described by Kato and Schimizu [41]. The reaction mixture (3 ml) consisted of 7.2 mM guaiacol, 11.8 mM hydrogen peroxide. Addition of 0.1 ml of crude enzyme extract initiated the reaction, which was measured spectrophotometrically (Genesys 10 vis) at 470 nm. Enzyme activity is expressed in terms of the change in absorbance per minute at 470 nm in the linear phase of the slope (Δ 470/ min/g fresh weight) immediately after the addition of substrate.

Statistical analysis

All results were subjected to one-way ANOVA and the means were compared according to the Student–Newman–Keuls (SNK) multiple range test ($P \leq 0.05$).

RESULTS

Selection of potential isolates and its characterization

Forty three bacterial isolates were obtained from barley rhizosphere in Taif province. These isolates were found to be tolerant to NaCl at a concentration as high as 10 %, and relatively different in their colony colors, appearance and texture. Different patterns of enzymatic activity were observed between the isolates (Table 1). No isolates showed a high activity for all enzymatic tests, but as a whole, at least some isolates were able to degrade the most substrates tested. Among the bacterial isolates, both isolate 20s and 34a were the most biochemically active, compared to the others. Isolate 20s showed high protease and chitinase production. A relatively high activity was detected for IAA production and cellulose hydrolysis. Its ability to dissolve phosphate compounds and degrade starch was moderate (Table 1). On the other hand, isolate 34a gave high activities for protease, chitinase and IAA production. A relatively high activity for phosphate solubilization and cellulose hydrolysis was observed. Isolate 34a showed moderate activity to degrade starch.The two selected bacterial strains shown a highly salt tolerant were subjected to extensive morphological, physiological, biochemical, nutritional and antimicrobial susceptibility testes (Table 2).

Strain20s has a creamy yellow colonies, rod shaped cells (1.4-1.7 X 0.6-0.8 µm) (Photo 1a), forming endospores. The cells were motile. The isolate required aerobic/anaerobic conditions for growth and grew in nutrient medium containing 0.0-25 % NaCl (Table 2). Optimal temperature was 35ºC. The temperature range was 15-50 ºC. pH range was between 6 up to 10.5. Gram reaction, methyl red, hemolytic activity, catalase, nitrate reductase and oxidase are positive. The isolateshowed negative for the following: Voges-Proskauer, indole production, H_2S production, citrate utilization and acid production from L-xylose, lactose, raffinose and D-fructose. It was able to utilize starch, gelatin, casein and chitin as a sole carbon source. It was unable to utilized cellulose and urea. The isolate tested against fifteen antibiotics listed in Table (3). The results obtained showed that our strain varied in its susceptibility to all antimicrobials used. It was resistant to ampicillin, flucloxacillin, bacitracin, penicillin G and polymyxin B (Photo 2a). The following antibiotics inhibit the growth of strain20s: tetracycline, chloramphenicol, levofloxacin, tobramycin, ofloxacin, norfloxacin, cefotaxime, imipenem, gentamicin and neomycin.

Strain 34a has a creamy faint yellow colonies, rod shaped cells (1.6-1.9X0.6-0.7) (Photo 1b), forming endospores and motile cells. The isolate grow aerobically in nutrient medium containing 0.0-20 % NaCl. Optimal temperature was 35 ºC. The temperature range was 10-50 ºC. pH range was between 6 up to 10. Gram reaction, hemolytic activity, indole production, catalase, nitrate reductase and oxidase are positive. While, it showed negative for the following reactions: methyl red, Voges-proskauer, H_2S production,

citrateutilization and acid production from galactose and lactose. It utilized various sugars including sucrose, D-xylose, L-xylose, D-glucose, raffinose, D-fructose, maltose and mannitol to produce acid. Decomposition of starch, casein cellulose, chitin and gelatin are positive. Among the antibiotics tested, the strain 34a was resistant to chloramphenicol, flucloxacillin bacitracin, pencillin G and polymyxin B (Photo 2b & Table 3).

Molecularcharacterization:

The aligned 16S rDNA sequences of 1247 bp showed base frequencies of A = 24.8%, C = 24.1%, G = 31.2% and T = 19.9%. Within 1247 nucleotides used for tree analyses, 1183 were constant and 64 were variables. From the variable sites, 30 were parsimony uninformative and 34 were informative under parsimony criterion. The consensus parsimony tree constructed showed consistency index (CI=0.92), homology index (HI =0.15), retention index (RI =0.93) and rescaled consistency index (RC = 0.85).

The data of the current strains were aligned with their counterparts of the related stains that were collected from the Genbank. They were as follows with their accession numbers in parenthesis: *B. subtilis* B1-33 (EU435361), *B. subtilis* PAB 1C8 C8(EU221345), *Bacillus tequilensis*strain IHBB (KR085936), *Bacillus subtilis inaquosorum* BGCS (JQ361055), *Bacillus sonorensis*strain CCMMB992(KF879304), *Bacillus aerius* BAB-2542 (KC443115) and *Bacillus licheniformis* DSM 13 (NC_006322). *Bacillus thioparans* strain BMP-1(NR_043762) and *Bacillus desertis*train ZLD-8 (NR_117383) were closely related to the target strains and therefore, they were used as an out group for tree rooting.

The three analytical methods executed a single tree with typical tree topology for all. The neighbor-joining tree was selected to be presented herein (Fig. 1). The tree indicated that *B. subtilis* YHSA20 clustered within the clade of *B. subtilis* with bootstrap support of 100%. *B*acillus sp. YHSA34 showed a sister relationship (bootstrap =97%) to different *Bacillus* species in a separate clade without deviation toward any of them.

The genetic distance showed the smallest values between *B. subtilis* YHSA20 and the other *B. subtilis* strains. *B*acillus sp. YHSA34 exhibited identical genetic relationship with both *B. licheniformis* M13 and *B. sonorensis* CCMMB987 (D =0.00) indicating that this strain could be either of any of the two species. Finally, the potent PGPR were identified as *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34, respectively.

Influence of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on barley growth

In pot experiments, many parameters were used to evaluate the effect of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on barely growth under salt stress. Results of *in vivo* tests revealed that application of *B. subtilis* YHSA20 and/or *Bacillus* sp. YHSA34 significantly increased barley in terms of fresh and dry weight. Barely fresh weight of microbial treated pots was insignificantly varied and higher than those of nontreated control at zero, 100 and 400 mMNaCl. It was significantly greater than those of nontreated plants at 200 and 300 mMNaCl. About, 21.5 and 25.6% increases of fresh weight were observed by *B. subtilis* YHSA20, respectively (Table 4). At all NaCl concentrations (0.0-400 mM), dry weight was varied significantly according to the microbial treatment. In case of *B. subtilis* YHSA20, about 48.4, 65.7 and 51.9 % increases were observed compared to their non-microbial treated control (0.0, 100 and 300 mMNaCl, respectively). An increase of 71.1, 57.4 and 89.7 was detected at 200, 300 and 400 mMNaCl, respectively using the dual inoculation by *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 (Table 4). Grain dry weight of all microbial treatments was varied insignificantly with those of their non-microbial treated control (Table 4). The higher value was observed at 200mM NaCl by *B. subtilis* YHSA20.

Effect of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on chlorophyll *a* and *b* content of barley leaves

Focusing on chlorophyll (*a*) content of barley leaves, a significant value was obtained by *B. subtilis* YHSA20 at all salt treatments compared to non-microbial treated plants (Table 5). The highest chlorophyll (*a*) induction was at 200 mMNaCl. About 18% increase in chlorophyll (*a*) was obtained by *B.subtilis* YHSA20 greater than the control. Among the microbial treatments at 400 mMNaCl, *Bacillus* sp. YHSA34 was the most chlorophyll (*a*) enhancer. On the other hand, the greatest chlorophyll (*a*) content obtained by the mixture of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 was at 300 mMNaCl. Our results indicated that *B. subtilis* YHSA20 enhanced chlorophyll (*b*) production in barley leaves at all tested salinity concentrations (Table 5). The highest value obtained at 200 mMNaCl. About 64.4 % increase in chlorophyll (*b*) was detected greater than the control. While, *Bacillus* sp. YHSA34 was the best inducer for chlorophyll (*b*) production in barley leaves at 400 mMNaCl.

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Effect of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on proline content and antioxidative compounds of barley leaves

Proline content varied significantly in all microbial treatments, in comparison with their nontreated control. The highest value obtained at all was by *B. subtilis* YHSA20 at 100 mM NaCl. Individual inoculation by *B. subtilis* YHSA20 enhancedthe proline production more two folds than in its control(Fig. 2). Moreover, about 98.8 % increase in the proline content was obtained by dual treatment of *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 compared to non-microbial treated control at 200 mM NaCl. Conversely, the greatest proline content was recorded for non-treated control at 300 mMNaCl.

Table 1.Biochemical activity and *in vitro* tests of forty three salt tolerant isolates from four different locations at Taif province for determination of PGPR activity.

Isolates	Protease production	Chitinase production	Phosphate solubilization	IAA production	cellulose hydrolysis	Starch hydrolysis
1h	+ + +	-	-	++	+ + +	+ +
2h	+ +	+	-	+	+	+
3h	+	-	-	-	+	-
4h	-	-	-	-	+	+
5h	+	-	+	+ + + +	-	-
6h	+ + +	-	-	-	+ +	+
7s	+ + +	+	-	-	+ +	+
8s	+ + +	+	-	-	+	-
9s	+ + +	+ +	+	+	+	-
10s	+	-	-	-	+	-
11s	-	+	-	-	-	+
12s	+	+	-	-	-	+
13s	+ + +	-	-	-	+ +	-
14s	-	-	-	-	-	+
15s	+ + + +	+	+	-	+	++
16s	++	-	-	-	+	+ +
17s	+	+	+	-	+ + +	+ + +
18s	+	-	-	-	+ +	+
19s	-	-	-	-	-	+
20s	+ + + +	+ + + +	+ +	+ + +	+ + +	+ +
21s	+ +	-	-	-	+ + +	+ + + +
22 sh	+ + +	-	-	-	+ +	+ +
23 sh	-	-	-	+ + + +	+	-
24 sh	+ +	+	+	+ + +	+ + +	+ + + +
25 sh	-	-	-	-	+	+ + + +
26 sh	+ + +	-	-	-	+ +	+ +
27 sh	+	-	-	-	+	+
28 sh	-	-	-	+ + + +	-	-
29 sh	+	+	-	-	-	-
30 sh	-	-	-	-	+	+
31a	+ + +	-	-	-	+ + +	+ +
32a	+ + + +	-	-	-	+ + + +	+ +
33a	+ + +	-	-	-	+ + +	+ +
34a	+ + + +	+ + + +	+ + +	+ + + +	+ + +	+ +
35a	+ +	+ + +	-	-	-	+
36a	+	-	+	-	-	-
37a	+ +	-	-	-	+ +	+
38a	+ + + +	-	-	-	+ + +	+
39a	+	-	+	-	+	-
40a	+ + +	-	-	+	+ +	+
41a	+ + +	+ + + +	-	+ + +	+ +	+ +
42a	+ +	-	-	-	+ +	-
43a	+ +	-	-	+	+ + +	+ + +

Letter indicated the location of isolation: Alhada (h); Ashayrah (s); Al Abar (a); Shogsan (sh).
Biochemical activity: (-) no ; (+) low; (+ +) moderate; (+ + +) relatively high; (+ + + +) high.

Concerning on non-treated controls, an increase in total phenolic compounds was recorded with increasing NaCl concentration (Fig. 3).While, total phenols varied insignificantly in all microbial treatments in comparison with their non-treated control except at 400 mM NaCl (Fig. 3). At the higher salinity concentration (400 mM) both *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 reduced barley induction of the phenolic compounds production, they caused more 55% reduction than in their non-treated control.

Peroxidase activity in barley leaves varied insignificantly in all microbial treatments, in comparison with their control (Fig. 4).The highest peroxidase activity obtained *B. subtilis* YHSA20 at 200 mMNaCl. It was 84.5 % greater than the control. However, a dramatic reduction was observed when NaCl concentration increased. A noticeable increase in peroxidase activity was obtained by the individual inoculation with *Bacillus* sp. YHSA34 from 200 to 400 mM NaCl.

Table 2.Morphological, biochemical and physiological characteristics of the selected halotolerant bacteria: strain 20s and 34a.

Characteristic	Strain 20s	Strain 34a
Pigmentation	Creamy yellow	Creamy faint yellow
Cell shape	Rods	Rods
Length	1.4-1.7µm	1.6-1.9µm
Diameter	0.6-0.8µm	0.6-0.7µm
Growth at:		
0.0% NaCl	+	+
10% NaCl	+	+
15% NaCl	+	+
20% NaCl	+	+
25% NaCl	+	-
30% NaCl	-	-
Motility	+	+
Soprulation	+	+
Anaerobic growth	±	-
Temperature range	15- 50ºC	15- 50ºC
pH range	7-10.5	6-10
Gram reaction	+	+
Methyl red	+	-
Voges-proskauer	-	-
Hemolytic activity	+	+
Indole production	-	+
H_2S production	-	-
Citrate utilization	-	-
Catalase	+	+
Nitrate reductase	+	+
Oxidase	+	+
Acid production from		
Sucrose	+	+
Galactose	+	-
D-xylose	+	+
L-xylose	-	+
Lactose	-	-
D- glucose	+	+
Raffinose	-	+
D-Fructose	-	+
Maltose	+	+
Mannitol	+	+
Decomposition of:		
Starch	+	+
Gelatin	+	+
Casein	+	+
Cellulose	-	+
Chitin	+	+
Urea	-	+

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Table 3. Antibiotic susceptibility ofthe selected halotolerant bacteria: strain 20s and 34a.

Characteristic	Strain 20s	Strain 34a
Ampicillin	-	+
Tetracycline	+	+
Chloramphenicol	+	-
Levofloxacin	+	+
Flucloxacillin	-	-
Tobramycin	+	+
Ofloxacin	+	+
Norfloxacin	+	+
Cefotaxime	+	+
Imipenem	+	+
Bacitracin	-	-
Penicillin G	-	-
Polymyxin B	-	-
Gentamicin	+	+
Neomycin	+	+

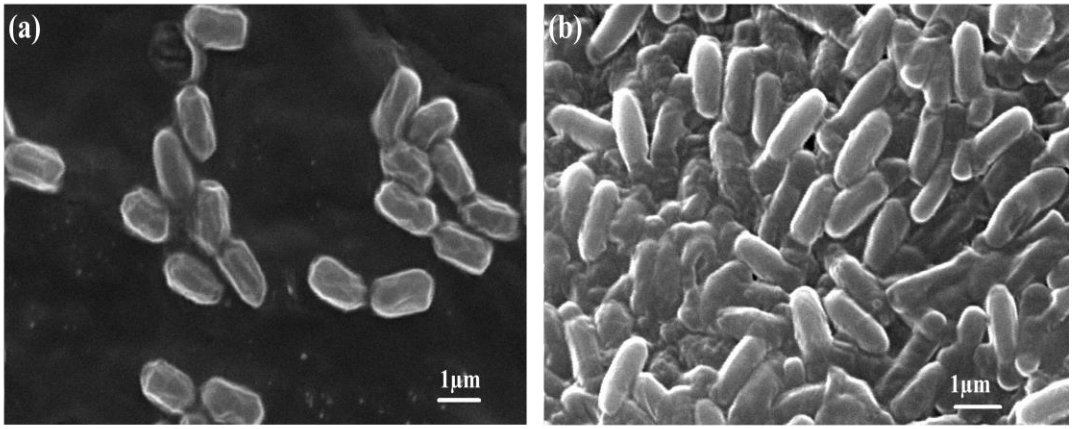

Photo 1.Scanning electron microscopy for (a) strain20s and (b) strain 34a.

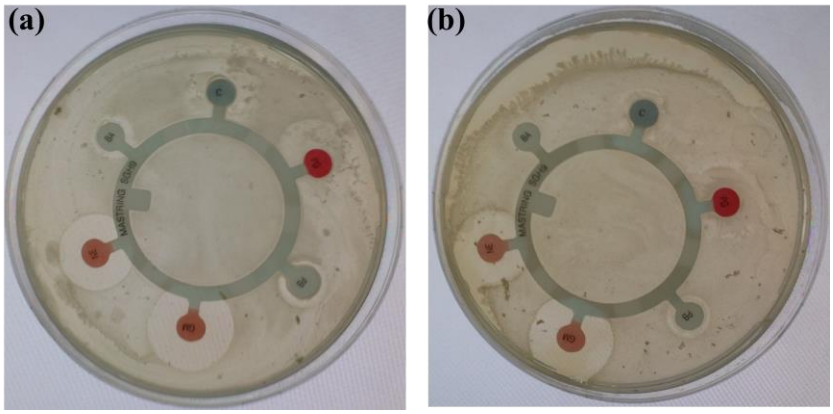

Photo 2. Comparison of the results of the antibiotic sensitivity assay for (a) strain20s and (b) strain 34a. against bacitracin (BA), chloramphenicol (C), penicillin (G), polymyxin (B), gentamicin (GM) and neomycin (NE). Different inhibition zones formed according to antibiotic and the strain.

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Table 4. Influence of single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on fresh weight (FW), dry weight (DW) and grain dry weight (G. DW) of barley.

Treatment	Salinity (NaCl) concentration (mM)														
	0.0			100			200			300			400		
	FW	DW	G. DW	FW	DW	G. DW	FW	DW	G. DW	FW	DW	G. DW	FW	DW	G. DW
Control	14.0 [a]	3.1 [b]	1143 [a]	17.1 [a]	3.5 [b]	1160 [a]	16.7 [b]	4.5 [bc]	1156 [a]	22.3 [b]	5.4 [b]	1073 [a]	20.4 [a]	3.9 [c]	1033 [a]
B. subtilis YHSA2	14.1 [a]	4.6 [a]	1180 [a]	17.6 [a]	5.8 [a]	1206 [a]	20.3 [a]	5.4 [b]	1404 [a]	28.0 [a]	8.2 [a]	1130 [a]	21.3 [a]	6.1 [b]	1123 [a]
Bacillus sp. YHSA34	14.5 [a]	3.0 [b]	1120 [a]	19.1 [a]	4.0 [b]	1156 [a]	17.9 [b]	4.0 [c]	1143 [a]	18.1 [c]	5.5 [b]	1156 [a]	22.4 [a]	5.4 [b]	1196 [a]
YHSA20 + YHSA3	15.7 [a]	3.6 [b]	1083 [a]	19.8 [a]	5.2 [a]	1131 [a]	18.0 [b]	7.7 [a]	1227 [a]	19.7 [c]	8.5 [a]	1133 [a]	20.7 [a]	7.4 [a]	1163 [a]

Different letters on the column for each parameter are differ significantly at p≤ 0.05.
Fresh weight, dry weight and grain dry weight are expressed in g/plant.

Table 5. Influence of single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*) content in barley leaves.

Treatment	Salinity (NaCl) concentration (mM)									
	0.0		100		200		300		400	
	Chla	*Chlb*	*Chla*	*Chlb*	*Chla*	*Chlb*	*Chla*	*Chlb*	*Chla*	*Chlb*
Control	183 [b]	21.7 [b]	198 [b]	54.4 [b]	226 [b]	65.6 [b]	201 [b]	53.9 [b]	202 [b]	58.4 [b]
B. subtilis YHSA20	230 [a]	77.0 [a]	242 [a]	80.4 [a]	267 [a]	107.9 [a]	234 [a]	67.0 [a]	224 [a]	69.5 [b]
Bacillus sp. YHSA34	183 [b]	22.3 [b]	190 [b]	30.3 [c]	191 [d]	27.9 [c]	233 [a]	73.9 [a]	224 [a]	85.9 [a]
YHSA20 + YHSA34	188 [b]	25.7 [b]	201 [b]	47.2 [b]	206 [c]	48.5 [b]	213 [a]	61.2 [a]	194 [a]	40.2 [c]

Different letters on the column for each parameter are differ significantly at p≤ 0.05.

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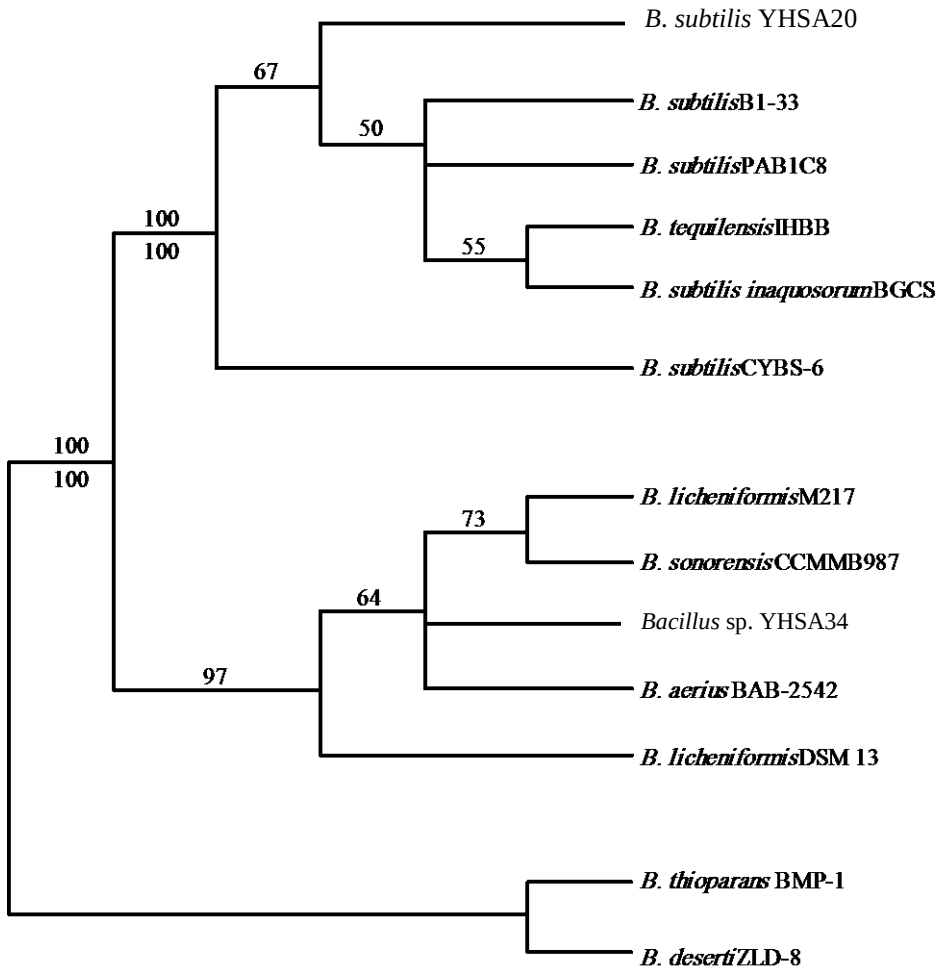

Fig. 1.The neighbor-joining tree constructed from 1247 bp of 16SrDNA for the different *Bacilli* strains and species studied. Values at nodes are the bootstrap supports when they were over 50%. The values below branches are the bootstrap supports for the MP and ML methods when they were available.

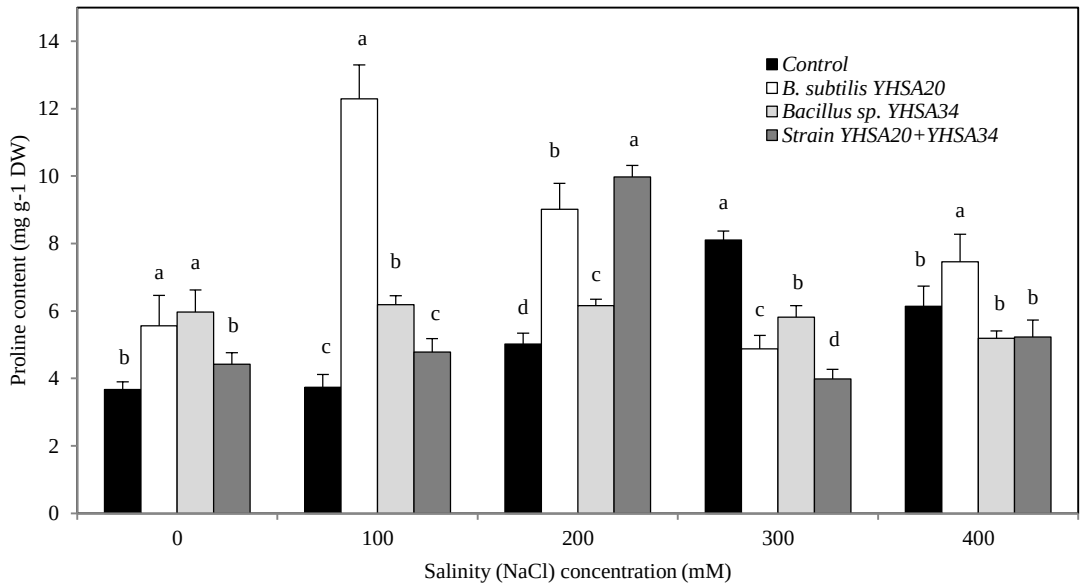

Fig. 2. Influence of single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on proline content of barley leaves.Different letters on the columns for each parameter (salinity concentration) are differ significantly at p≤ 0.05.

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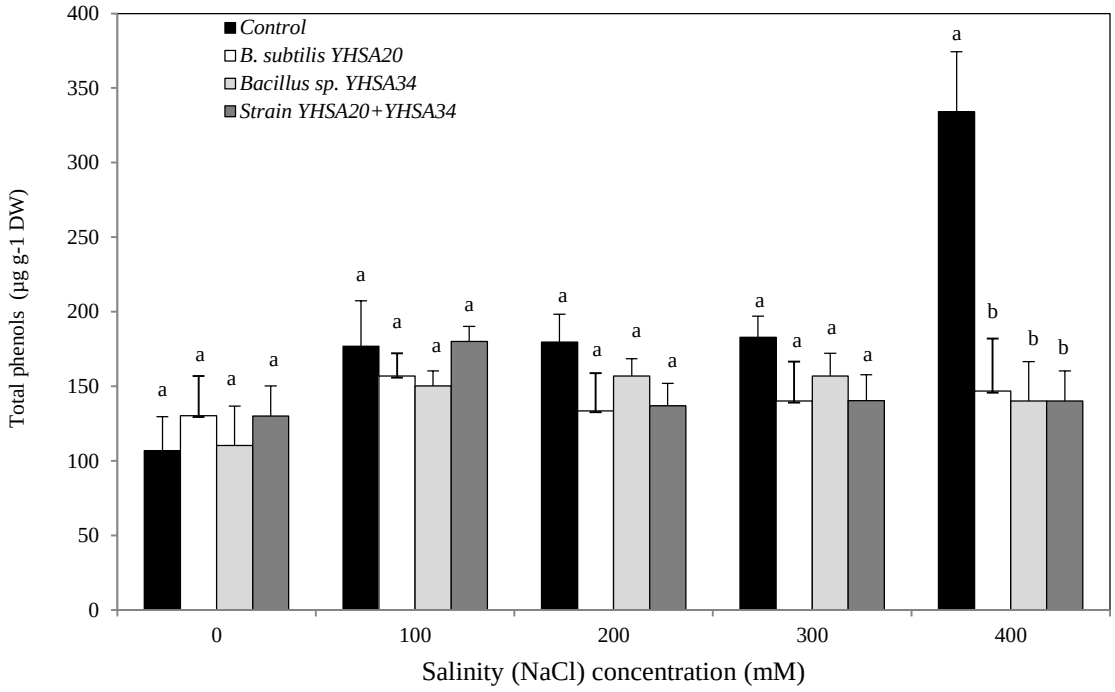

Fig. 3. Influence of single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on total phenol content of barley leaves.Different letters on the columns for each parameter (salinity concentration) are differ significantly at p≤ 0.05.

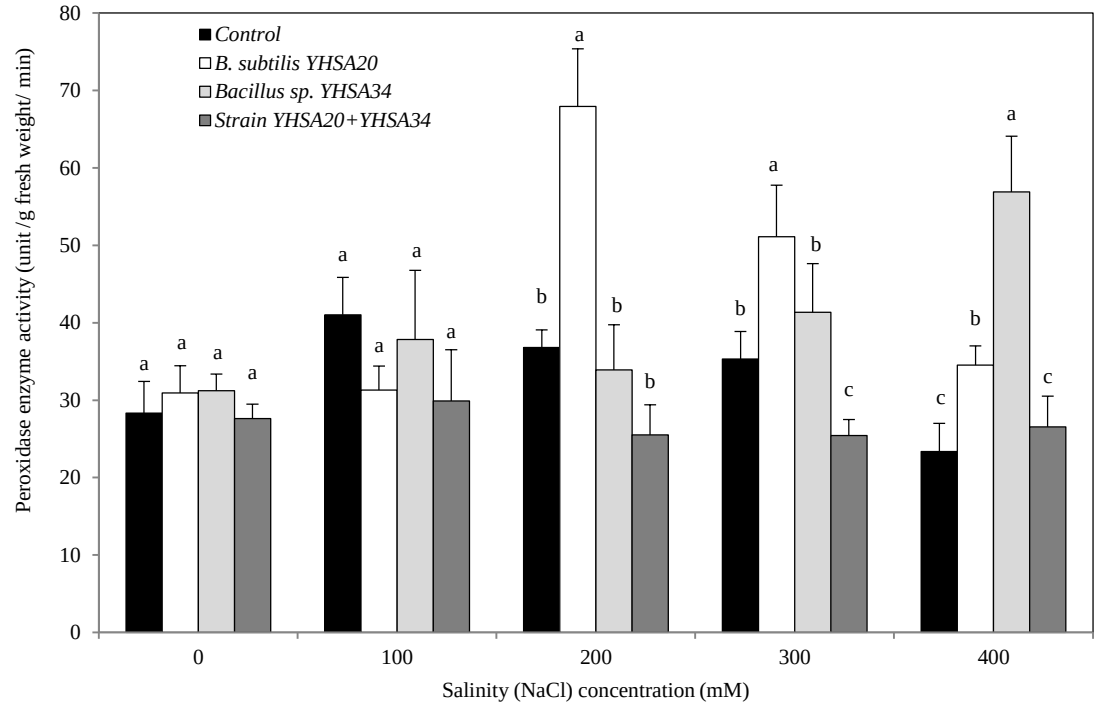

Fig. 4. Influence of single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 on peroxidase enzyme activity in barley leaves. Different letters on the columns for each parameter (salinity concentration) are differ significantly at p≤0.05.

DISCUSSION

Although barley (*Hordeum vulgare* L.) is regarded as salt tolerant among crop plants, its growth and development are severely affected by ionic and osmotic stresses in salt-affected soils. Salinity stress affects nutrient uptake [42] and metabolic activities in plant [43]. It adversely affects almost all stages of growth and development, including germination, growth and vigor of seedling, vegetative growth, flowering and fruit set and ultimately causing diminished economic yield and also quality of products [44,45]. However, the salinity effect magnitude is depending on plant species and level of salinity [46]. The harmful effect of salinity stress may be due to specific ion toxicity, disturbance in homeostasis of Na^+ and Cl^- ions, stomatal closure, and the increased production of ROS in chloroplasts [47, 48,49,50]. In the present study, salt stress treatments under low concentrations (0.0 to 300 mM of NaCl) increased barley fresh, dry weight, grain dry weight, chlorophyll, total phenols, proline content. A dramatically decrease was obtained with 400 mM of NaCl. Grain yield per plant was insignificantly reduced with increasing NaCl concentration. These results were in agreement with Ahmad *et al*. [51] who reported that increase of sodium chloride concentration reduced number of spikelts per spike, biomass per plant and grain yield per barely plant. Similarly, El Madidi*et al*. [52] showed that salinity stress decreased dry weight of barley roots and shoots. In this study, single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 enhanced all previous parameters except total phenols, compared with their controls. This clearly demonstrates that our selected bacteria canalleviate some of the debilitating effects of salt stress.In another studies, large number of PGPBs have been reported to provide tolerance to plants under different abiotic stress [7, 53,54,55,56]. Studies reported by Rojas-Tapias*et al*. [57] showed that *Azotobacter chroococcum*-inoculated maize plants had significantly higher biomass than their respective controls, under salt stress conditions. Plant growth promoting bacteria have been found to improve growth of tomato, pepper, canola, bean and lettuce under saline conditions [58, 59].Jha*et al*. [60] reported that the combination of *Pseudomonas pseudoalcaligenes*, an endophytic bacterium with a rhizospheric *Bacillus pumilus* was able to protect the *Oryza sativa*plant from abiotic stress by induction of osmo-protectant and antioxidant proteins. They stated also that plants inoculated with endophytic bacterium *P. pseudoalcaligenes* showed a significantly higher shoot biomass at lower salinity levels. While at higher salinity levels, a mixture of both *P. pseudoalcaligenes* and *B. pumilus* showed better response against the adverse effects of salinity.

Leaf chlorophyll concentration is an indicator of salt tolerance and responds to increasing salinity [61]. Our results are in agreement with Rojas-Tapias*et al*. [57] who reported that the inoculation with *Azotobacter chroococcum* C5 and C9 enhanced the content of chlorophyll revealing a positive effect on growth and plant development. Notably, in the present study, at NaCl concentrations 0, 100, 200 and 400 mM, *B. subtilis* YHSA20significantly increased the proline content in barley leaves, indicating that this strain could generate some kind of stress on plant growth. Similarly, Szidericsz*et al*. [6] reported that *Arthrobacter* sp. EZB4 and *Bacillus* sp. EZB8 increased the proline content in pepper plants even in the absence of abiotic stress.

Many researches with application of PGPRs have shown significant increase in plants of several of plant defence-related enzymes, like peroxidase and phenolics[8, 62].Similarly, Singh *et al*. [63] showed that the inoculation of chickpea seeds with *Pseudomonas fluorescens* and *P. aeruginosa*, singly or in combination, induced the synthesis of specific phenolic acids (gallic, ferulic, chlorogenic) and increased total phenol content at various stages of plant growth. Also, the total phenolic content in marigold plants was two-fold higher in singly-inoculated or co-inoculated treatments than in controls [64]. Our results were not in agreement with these findings. The results demonstrated that single and dual inoculation with *B. subtilis* YHSA20 and *Bacillus* sp. YHSA34 reduced total phenolic compounds formation in barley leaves. That means that our strains acted as a first line defense to protect barely against the detrimental effects of salt.Single inoculation with *B. subtilis* YHSA20 or *Bacillus* sp. YHSA34 could elicit antioxidant responses against salt stress in barley leaves, as evidenced by increased activities of peroxidase enzyme. Our results are in agreement with Chakraborty *et al*. [65] who reported that *Bacillus cereus* elicited antioxidant responses in *Cynodondactylon* against salt stress by increasing peroxidase activity. Studies achieved by Gururani*et al*. [66] reported that different ROS pathway genes enhanced mRNA expression under salt stress in PGPR inoculated potato plants. In conclusion, inoculation with the PGPR *Bacillus subtilis* YHSA20 and *Bacillus* sp.YHSA34 could serve as a useful tool for alleviating the harmfull effect of salinity stress and enhance plant growth of barley, grown under saline conditions, and could be appropriate bioinoculants under such habitats.

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