Original Research Article

Alleviation of salt stress on wheat (*Triticum aestivum L.*) by plant growth promoting bacteria strains *Bacillus halotolerans* MSR-H4 and *Lelliottia amnigena* MSR-M49.

Abstract

The plant growth-promoting rhizobacteria (PGPR) application could reduce the use of synthetic fertilizers and increase the sustainability of crop production. Halophilic bacteria that have PGPR characteristics can be used in different environmental stresses. Two different strains isolated, purified, characterized as a PGPRs and phylogenetic identification using 16sRNA which was revealed to be closest matched at 99% with *Bacillus halotolerans* and *Lelliottia amnigena*. The isolates possessed properties of plant growth promoting bacteria; Exopolysaccharides production (EPS), *Bacillus halotolerans* had the ability to Nitrogen fixation, two strains have the ability to P-solubilization, and production of indole acetic acid (IAA). Furthermore, the strains were tested in two experiments (Pots and a Field). Strains that possessed the four traits associated with PGPR significantly increased the plant height, straw dry weight (DW g plant^{-1}), spike number, 1000 grain DW recorded 31.550 g with *Lelliottia amnigena* MSR-M49 compared to un-inoculated and other strain in field, grain yield recorded 2.77 (ton fed^{-1}) with *Lelliottia amnigena* as well as N% and protein content in grains recorded 1.213% and 6.916 respectively with inoculation with *Lelliottia amnigena*, also, spikes length, inoculated wheat show reduction in both proline accumulation in shoots and roots especially with *Lelliottia amnigena* recorded 2.79 (mg g^{-1}DW), inoculation significantly increased K in root-shoot, K/Na in root-shoot, and reduced Na in root-shoot compared with control. This confirmed that this consortium could provide growers with a sustainable approach to reduce salt effect on wheat production.

Key words: wheat; salinity; PGPRs; 16sRNA; *Bacillus halotolerans*; *Lelliottia amnigena,* nitrogen fixation.

Introduction

Soil salinization is defined as process of increasing dissolved salts in the soil profile. At a global level, the total amount of saline soils is around 15% in arid and semi-arid regions and approximately, 40% in irrigated lands **[1].** It severely affects soil health,

which in turn affects crop productivity **[2].** The accumulation of salt in soil reduces the soil water potential and affects water and nutrient uptake by plant roots **[3],** thereby directly affecting the growth and diversity of organisms and plants. In plants, a high soil salinity conditions cause ionic and osmotic stress that adversely affects the functioning of various biochemical processes **[4].** Further, excessive sodium and chloride concentrations adversely affect the energy production and physiology of the plants by interfering with various enzymes activities **[5].** Salt stress results in a significant decrease in productivity of salt-sensitive and salt-tolerant crops. Wheat is the main staple food crop of Egypt. Wheat (*Triticum aestivum* L.) is one of the three major cereals source of energy, renewable resource for food, feed and industrial raw material, protein and fiber source in human diet, staple food crop for more than one-third of the world population **[6].** Most the cereal crops have low salinity or salt stress thresholds. For example wheat can tolerates salinity up to 6 dSm^{-1}, while the salinity threshold for maize is three times less (approximately 2 dSm^{-1}) **[7].** Worldwide agriculture is currently facing big challenges posed by the increase in global population and climate change, and plant growth-promoting bacteria (PGPB) are becoming an important alternative for sustainable crop production **[8, 9].** In addition, it has been demonstrated that beneficial microorganism play a significant role in alleviating salt stress in plants, resulting in increased crop yield and reduce salt stress in maize and wheat by approximately 50% **[10].** Under conditions of salinity, crop plants face disorder in several metabolic pathways, such as those related to photosynthesis, respiration, redox system homeostasis, phytohormone regulation, and carbohydrate and amino acid synthesis, which leads to reduced seed germination, plant growth and yield **[11, 12, 13].**

Lugtenberg and Kamilova [14] and Ahmad *et al.*, [15] demonstrated that a group of Plant-growth-promoting (PGP) bacteria enhance the growth of plants in two ways directly by produce some compounds indole acetic acid, siderophore, etc., solubilize minerals and degradation of organic matters for easy uptake by plants and for their own use, fixed atmospheric nitrogen that enhance the bioavailability of iron and synthezise phytohormones; cytokinins, auxins and gibberellins which have beneficial roles in various stages of plant growth **[16, 17]** or Indirectly, they aid in decreasing or inhibiting the detrimental effects of pathogenic organisms by enhancing the host resistance to pathogenic organisms by antibiotic production [**18, 19].** *Bacillus* spp. have been identified as the predominant communities **[20]** and resulted in a 40%

increase in crop yield **[21]** and it have been commercialized for improving crop production **[22, 23],** which is an eco-friendly approach to sustainable agriculture [**13, 24, 25].** Exopolysaccharide (EPS) in the rhizosphere soil binds Na^+ and inhibits Na^+ transport into plant root cells **[26].** Inoculating wheat seedlings with EPS producing *Bacillus insolitus* MAS17 and certain other *Bacillus* spp. covers the root zones with soil sheaths, enhancement of the K^+/Na^+ ratio in plants **[27, 28],** elevated levels of N, P, K, Ca, Mg, S, Mn, Cu, and Fe produced, reduce the toxic effects of salinity by inhibiting lipid peroxidation **[28]**, specifically that of oleic, linoleic, and linolenic acids as well as phospholipids **[29]** in plants grown in salt affected soils. **[30, 31]** Inoculation of chickpea plants with salt tolerant *B. subtilis* RH-4 improves seed germination, synthesis of photosynthetic pigments, carbohydrates, proteins and osmolytes, such as proline, glycine betaine, choline and enhanced plant growth. In addition, some of the secondary metabolites, such as gallic acid, caffeic acid, syringic acid, vanillic acid, ferulic acid, cinnamic acid, and quercetin, are increased in plants associated with bacteria, which allows plants to tolerate salt stress **[32].**

Entophytes may promote plant growth and yield, tolerance to biotic and abiotic stresses **[33, 34].** This group of bacteria does not visibly harm the host plant and can be isolated from surface-disinfested plant tissues or extracted from inside the plant **[35].** *Enterobacter* and *Bacillus* are among the most frequently isolated native entophytes found in the microbiota of several plant species **[36, 37].**

Over the past few years, a number of *Enterobacter* sp. and close relatives in the family Enterobacteriaceae showing PGP under abiotic stress have been also characterized. For example, *Enterobacter cloacae* SBP-8 (formerly *Klebsiella* sp. SBP-8), which induced systemic tolerance in wheat under salt stress **[38],** *E. cloacae* UW5, which was able to produce high-levels of indole-3-acetic acid (IAA) **[39]**.

Materials and methods

Sites of bacterial isolation

Bacteria were isolated from the rhizosphere soil of wheat plant grown on saline soil in two different sites; Sahl El-Hussinia Governorate at (28°2.033' N; 1°39.578' E) and from El-Arish region, North Sinai Governorate at (31°07'26.2"N; 33°49'53.9"E) Egypt. The rhizobacteria were isolated according to the dilution plate technique adopted from **Baig *et al.* [40].** The rhizosphere soil samples were collected by vagaries shaking of the root system in 50 ml plastic tube. 0.5g of the rhizosphere was

diluted with autoclaved saline solution (0.9% NaCl) and serial dilution was prepared and plating on LB agar plates as described before **[41].** The plates were incubated at 28°C until appearance of bacterial colonies. Individual colonies were picked and streaked on LB plates for further purification. The purified strains were stocked with 20% glycerol and kept at -80°C.

Identification and taxonomic classification of the isolated bacteria

Purified strains were revived on LB agar plates from which a single colony was used to inoculate 10 mL of LB medium and incubated for 16 h at 28°C with shaking 220 rpm. The cells were then centrifuged at 12.000 × g and the pellet was used for genomic DNA extraction using a DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

For the identification of the bacterial isolates, bacterial universal primers were used for amplification of the 16S rRNA gene using PCR master mix (Promega): Universal primer sets 27F and 1492R (27F primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R primer 5'-TAC GGY TAC CTT GTT ACG ACT T-3'). PCR amplification of 16S rRNA genes was performed in a thermal cycler (Bio-Rad), with the following PCR conditions: 95°C for 1 min, 30 x (95°C for 30 sec, 55°C for 45 sec, 72°C for 90 sec), and a final extension step for 5 minutes at 72°C. PCR products were cleaned and purified using gel purification kit (sigma) and sequenced using ABI 3730xl DNA Analyzer (Applied Biosystems).

The resolved 16S rRNA gene sequences of the bacterial isolates were compared with known sequences listed in the GenBank nucleotide sequence database using the online software BLAST of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) **[42]**. The 16S rRNA gene sequences of the bacterial isolates in this study have been deposited in the GenBank database. Multiple alignments of the nucleotide sequences were performed with the program MUSCLE **[43].** The phylogenetic tree was constructed by the Neighbor-Joining method **[44],** based on the Kimura 2-parameter model **[45],** with bootstrap analysis (1,000 replications) using the software MEGA (version 7) **[46].**

Biochemical characterization of the isolated bacterial strains:

Indole acetic Acid (IAA) production

Bacterial strains (*Bacillus halotolerans* MSR-H4 and *Lelliottia amnigena* MSR-M49) were tested for the production IAA, as described by **[47]**, 1ml (10^7 cfu ml^{-1}) from

suspensions these bacteria was inoculated in Nutrient Broth medium supplemented with 1 gL^{-1} tryptophan and incubated at 30^0C on a shaker at 200 rpm for 72h, after incubation period, Bacterial cells were centrifuged at 8000 rpm for 10 min, 0.5 ml of the supernatant was mixed with 2 ml of the Salkowski Reagent, the optical density was measured and recorded at 540 nm using Spectrophotometer .

Exopolysaccharides production (EPS).

For the determination of EPS production, MSR-H4 and M49 was inoculated into conical flasks containing 100 ml of nutrient broth supplemented with 1% of sucrose. The inoculated flasks were incubated at 30 ± 2^{o}C on a rotary shaker at 200 rpm for 72 h. After incubation, the bacteria broth was centrifuged (3500 x g) and the supernatant was mixed with two volumes of acetone. The polysaccharides developed were collected by centrifugation at (3500 x g) for 30 min. The EPS was washed with distilled water and acetone alternately, transferred onto a filter and weighed after overnight drying at 105°C **[48].**

Acetylene Reduction Assay (ARA)

Bacteria was grown in nitrogen deficient medium for three days after we tested it to nitrogen fixing activity on Gas Chromatograph according to methods described by **Hardy *et al.*, [49].**

Assessment of Phosphate Solubilization

Phosphorus solubilizing activities of the bacterial isolate was examined using Pikovskaya's (PVK) as described by **[50].** By adding 1 ml of cultures bacterial culture with (10^7 cfu ml^{-1}) on Pikovskaya's (PVK) media plates supplemented by 5 g of tricalcium phosphate (TCP) as sole phosphorus source, then plates were incubated at 30°C for 7 days. The clear zone as indicator the solubilization of phosphate was recorded.

Pathogenicity assay

As the soil bacteria might carry a virulence factors could have a thread in plant heath, we examined if two isolated strains have any pathogenicity effect on different plant under greenhouse condition e.g. *Arabidopsis thaliana*, and tomato plants. For the bacteria we use *Escherichia coli* DH5α as negative control and *Pseudomonas*

syringae tomato DC3000 as a Positive control. We sprayed the plants with bacterial

Parameter	2.5 dSm^{-1}	4 dSm^{-1}	6 dSm^{-1}	Field (5.2 dSm^{-1})

inoculum (10^7 cfu ml^{-1}) and we monitor the plant for 4 weeks for symptom developing if any.

Soil analyses

The pH was directly measured in the water extracted sample 1:5 w/v using a glass electrode pH meter (Orion Expandable ion analyzer EA920). Electrical conductivity measurements were run in 1:5 w/v using EC meter (ICM model 71150). The cations analyzed in saturation soils sample experiment extracts are Ca^{++}, Mg^{++}, K^+ and Na^+ and the anion SO_4^{--} (Meq l^{-1}) were estimated as described by **Richards [51]**, while the anions CO_3^{--}, HCO_3^- were estimated by titrating with $KHSO_4$ (N/50) using phenolphthalein indicator for the former and bromocrysol green for the latter [51]. Chlorides Cl^- was determined by titration (5 ml of samples) against standard solution of sliver nitrate as conducted by Moher's methods [52]. Total nitrogen was determined as described by **Chapman and Parker [53].** For determination of N, K-contents expressed of samples were dried and 0.2 g were incubated in 5 ml H_2SO_4 and 1 ml perchloric acid in a conical flask for 24 h as described by **Chapman and Parker [53].** The digested materials were completed to 50 ml H_2O and then distilled by a micro-Kjeldahl method and the nitrogen concentration of distillate was determined by titration against 0.02 normal H_2SO_4 as conducted by **Black *et al*. [54].** Phosphorus concentration of samples was determined calorimetrically as described by **Snell and Snell [55]**. Potassium contents were determined for the digested solution by using flam photometer (No, 712700 REG. DES No, 866150) as described by **Jackson [56]**. The results of the Soil characterization of pots and field experiments were reported in Table 1.

Table 1: Soil characterization of the pots and field experiments.

Some physical properties				
Particle size distribution				
Clay%	54.6	54.0	53.6	50.1
Silt%	22.1	22.7	22.4	24.4
Coarse sand%	5.7	6.0	6.7	6.7
Fine sand%	17.6	17.3	17.3	19.3
Texture grade	Clayey	Clayey	Clayey	Clayey
pH (1:2.5 water suspension	7.8	8.1	8.6	8.4
EC (dSm^{-1} in soil paste extract)	2.4	4	6	5
Soluble cations, meq/L				
Ca^{++}	8.2	8.2	13.2	13.0
Mg^{++}	4.7	4.7	9.7	9.9
Na^{+}	10.7	26.5	36.5	26.9
K^{+}	0.41	0.43	0.39	0.5
Soluble anions, meq/L				
CO_3^{--}	0.0	0.0	0.0	0.0
HCO_3^{-}	5.3	5.5	5.0	5.5
Cl^{-}	8.8	18.6	28.9	23.6
SO_4^{--}	10.7	15.7	25.9	20.7
Available macro elements, ppm				
N	43.6	38.4	31.6	40.6
P	10.5	7.6	5.5	8.5
K	420	390	365	4.15

Experimental field trials

Field location and agriculture practice

The Egyptian cultivar winter Gemza 12 (*Triticum aestivum* L) was used in this study. The Gemza has good agronomic characteristics and developed by the National Wheat program, Field crop Research Institute, Agricultural Research Center (ARC), Egypt.

The evaluation impact of the salt-tolerant *Bacillus halotolerans* strain MSR-H4 and *Lelliottia amnigena* strain MSR-M49 on productivity of wheat plants under salt stress were assisted on in season, 2018-2019 under both greenhouse pots and fields experiments. The experiments were carried out at Sakha Agricultural Research Station, Kafrelsheikh, Egypt. The experiment was planned according to a randomized complete block design (RCBD) with three replications for each treatments -non-inoculated plant, inoculated with either MSR-H4 and MSR-M49, and dual inoculation with both strains. The wheat on both field and pots experiment were NPK fertilization according to stander agriculture practices recommended by Ministry of Agriculture. This includes urea as N source with the rate of 230 kg urea fed^{-1} and both phosphate and potassium with a rate of 100 Kg fed^{-1}. Agronomical data were recorded

at 75 day of sowing and at harvesting time. The used pots were about 35 cm in diameter and 40 cm in high filled with 8.5 kg clay soil and the field plot of greenhouse experiment was (100 cm x 100 cm) 1 m^2 with 3 replicates.

Bacterial inoculum preparation

Bacterial cultures MSR-H4 and MSR-49 at exponential phase ($6x10^7$ and $5x10^7$ cfu g^{-1}, respectively) were carried on (1:1) vermiculite: beat moss using Arabic gum as adhesive agent to form slurry. The slurry was then mixed with the seed until it was evenly coated. The coated seeds were lifted to dry in the shed for 60 minutes and planted in soil.

Agronomical parameters and Data collection

Plant biometrics was estimated at 75 day from sowing in pots this included measurement of total chlorophyll a cording to **Nornai, [57]**, dry weight of plant and plant height. After maturation and harvesting we collected data related to grain yield (g $plant^{-1}$, spike number $plant^{-1,}$ weight of 1000-grain and total protein in grains). While the effect of inoculation in field experiment estimated at the harvesting (plant height cm, straw dry weight (g $plant^{-1}$), spike number and (g $plant^{-1}$), 1000 grain dry weight (g), grain yield (ton fed^{-1}), N% was determined by Kjeldahel technique besides to protein in grains, spike length (cm), water content in both shoot and root (WC%), proline content in both in shoot and root (mg g^{-1}DW) **Bates *et al.* [58]**. Na, K in both shoot and root (mg g^{-1}DW), K in root (mg g^{-1}DW) **Wolf [59]**, followed by calculation of K^+/Na^+ in root.

Statistical analysis

The data collected during the experiment were analysis by using CoStat program version 6.303. By one variances (one ways) analysis (ANOVA). Differences at $p < 0.05$ were considered to be significant. The experiments were applied at three replicates.

Accession numbers

The 16S rRNA gene sequences of the two bacterial isolates in this study have been deposited at DDBJ/EMBL/GeneBank under accession numbers MN494097 and MN494098

Results

Isolation and Identification of Rhizobacteria

From wheat plant (*Triticum aestivum*) grow in two different saline soils in Egypt, a diverse number of bacteria strains were isolated. Hereafter we focused on

characterizing two top strains isolated from wheat rhizosphere (see Material and method). Based on the 16S sequence blast search using NCBI database, and the phylogenetic and taxon classification, we identified the first isolate from Sahl El-Hussinia Governorate as *Bacillus halotolerans* strain MSR-H4 accession no MN494097 and an isolate from El-Arish region, North Sinai Governorate identified as *Lelliottia amnigena* strain MSR-M49 with accession no. MN494098. *B. halotolerans* strain MSR-H4, were belonging to Firm cutes phyla with a highly aligned with the genera Bacillus, the blast search show high similarity with *Bacillus halotolerans* strain DSM 8802 with 99% identities. While the blast search with 16S rDNA of isolates MSR-M49, reveal high similarity with *Lelliottia amnigena* strain JCM1237 (NR_024642.1). The *Lelliottia amnigena* formally *Enterobacter amngena* belongs to Proteobacteria, family Enterobacteriaceae as showed in Figure 1. The phylogenetic relation of MSR-H4 and MSR-M49 are represented in the phylogenic tree (Figure 1) where two group are presented: Group A (Bacillus group) where the MSR-H4 are clustered with different bacillus strains and closed to *B. halotolerans* and Group B Enterobacteriaceae group, where MSR-M49 strains are clustered with different Enterobacter and *Lelliottia* species.

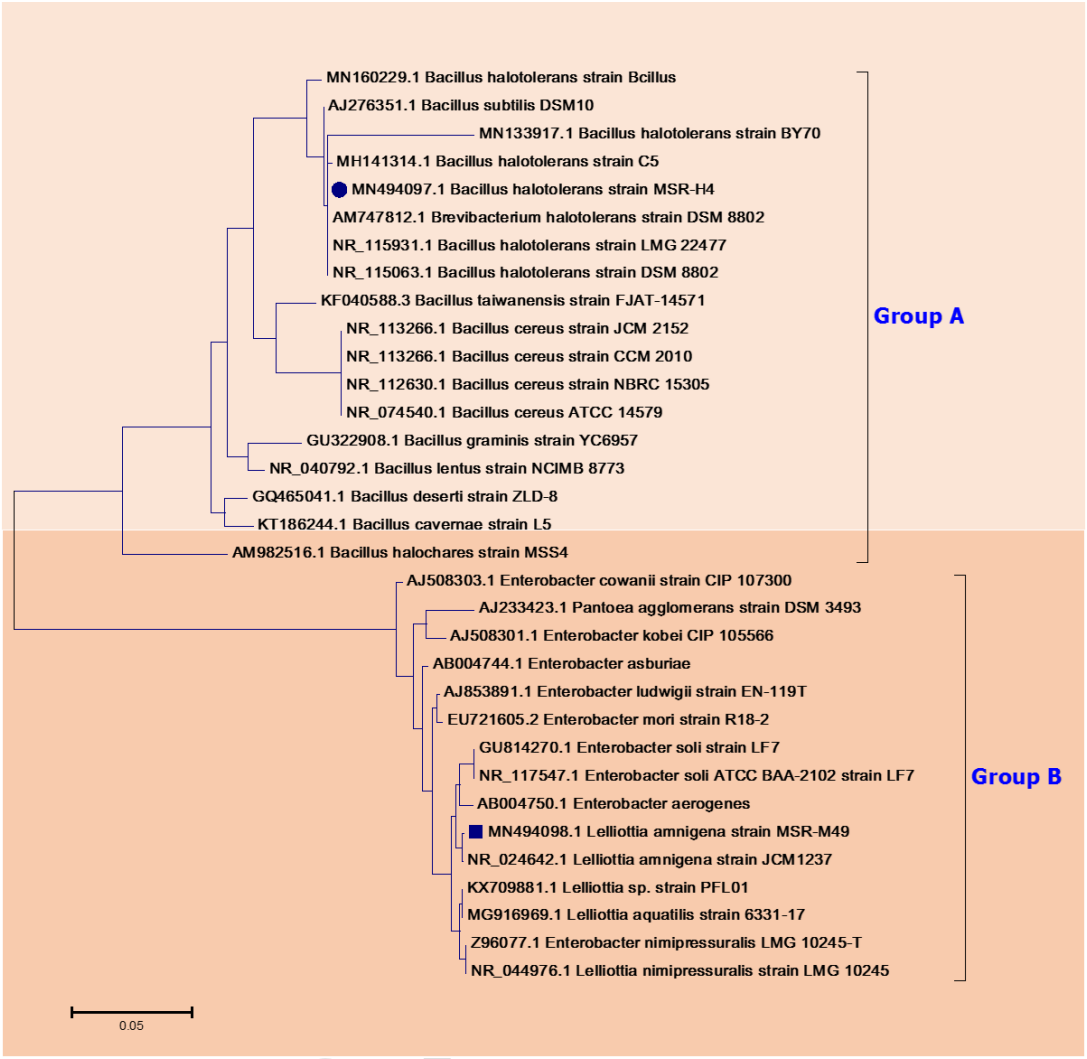

Figure 1: Phylogenetic tree of isolated rhizosphere bacteria

Phylogenetic tree of rhizosphere bacteria based on 16S rRNA gene sequence comparison. Evolutionary relationships of the bacterial strains group A *Bacillus* and Group B Entrobacteraciease inferred using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method. There were a total of 1177 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [**60**] Group with highlight of *Bacillus halotolerans* strain MSR-H4 (●) and *Lelliottia amnigena* strain MSR-M49 (■). GenBank accession numbers are presented for each strain. All positions containing gaps and missing data were eliminated.

Characterization of the bacterial isolates

The biochemical characterization of the MSR-H4 and MSR-M49 confirmed their effectiveness as plant growth-promoting microbes (Table 2). Both strains MSR-H4 and MSR-M49 were able to produce IAA as well solubilize the tricalcium phosphate. Interestingly, MSR-H4 has the capacity to fix the atmospheric nitrogen while the MSR-M49 is impaired. Both strains are producer of exopolysaccharides with similar rate. *Bacillus halotolerans* MSR-H4 strains had the ability to fixed Nitrogen while *Lelliottia amnigena* strain MSR-M49 not detected.

Table 2: Biochemical properties of the isolated strains

Strain	Indole acetic acid (IAA) (µgml^{-1})	EPS (g/100 ml)	N_2-activity (µ moles C_2H_4 /ml/h)	P. solubilizing
Bacillus halotolerans MSR-H4	66.0	4.2	6.71	+
Lelliottia amnigena MSR-M49	14.45	5.2	*n.d*	+

Pathogenicity assay

In comparison to the phytopathogen *Pseudomonas syringae* pv. tomato DC3000, none of the tested strains MSR-H4 , MSR-M49 and *E. coli* DH5α show any characteristic symptom of DC3000 including hypersensitive response (HR) on the leaves.

Agronomical impact of the isolated strains on wheat growth

Green house, pot experiment

The two bacterial strains were used in two successful seasons; in pots (2017-2018) and field (2018-2019) experiments in inoculation of wheat plants under soil salinity effect. From the pots experiment (Table 3), the chlorophyll content decreased with increasing salinity levels. While the influence effect of inoculation with *Bacillus halotolerans* MSR-H4 and *Lelliottia amnigena* MSR-M49 strains on chlorophyll content of wheat at 70 days of sowing showed positively increased compared with control.

Table 3: The effect of bacteria inoculation on plant growth (Pots experiment)

Treatments Main (Salinity dSm^{-1})		Total chlorophyll (mg g^{-1} fresh weight)	Plant height (cm)	Dry weight of plant (g)
2.5 dSm^{-1}		43.94	79.04	7.94
4.0 dSm^{-1}		40.71	76.55	7.36
6.0 dSm^{-1}		38.38	74.61	6.62
L.S.D. 0.05		1.32**	1.85**	0.39**
Sub main (Bacteria)				
control		34.98	73.50	4.52
MSR-M49		43.29	76.98	8.31
MSR-H4		40.28	76.28	6.85
Duel inoculum		45.48	80.18	9.54
L.S.D. 0.05		1.33**	1.38**	0.3**
Interaction				
2.5 dSm^{-1}	Control	38.62	75.38	4.78
	MSR-M49	46.69	80.22	9.16

	MSR-H4	42.35	78.87	7.28
	Duel inoculum	48.10	81.70	10.53
4 dSm^{-1}	Control	34.17	72.96	4.69
	MSR-M49	42.80	76.73	8.59
	MSR-H4	40.41	76.05	6.83
	Duel inoculum	45.47	80.44	9.33
6 dSm^{-1}	Control	32.15	72.15	4.09
	MSR-M49	40.38	73.99	7.18
	MSR-H4	38.09	73.92	6.45
	Mixed inoculum	42.88	78.38	8.75
L.S.D. 0.05		*n.s*	*n.s*	0.51**

Mixed inoculation with the two strains was the best compared with other treatments at all the salinity levels 48.10, 45.47 and 42.88 at 2.5, 4.0 and 6.0 dSm^{-1} respectively. The duel inoculation do as synergistic effect by N-fixation and PGPR activity which increased plant growth characterization and so improved the healthy status of plants. The wheat plants height were decreased with increasing salinity. Inoculation with MSR-H4 and/ or MSR-M49 strains increased wheat highest as reported in Table 3. It had showed increased in wheat height with inoculation compared with control under all salinity levels tested and the duel inoculation with the both strains were the greatest value. Dry weight of wheat plants under salinity levels were estimated in pots experiment. Single or dual inoculation gave the best dry weight compared with control at all salinity levels. The duel inoculation was the greatest dry weight at all salinity levels 10.53, 9.33 and 8.75 g $plant^{-1}$ at 2.5, 4.0 and 6.0 dSm^{-1} respectively.

Spike number ($plant^{-1}$), grain yield (g $plant^{-1}$), 1000-grain weight (g) and total N% in grain were estimated in the pots experiment at the harvest in Table 4.

Table 4: The effect of bacteria inoculation on wheat yield (Pots experiment)

Treatments **Main (Salinity dSm^{-1})**	**Grain yield (g $plant^{-1}$)**	**Spike number $plant^{-1}$**	**1000-grain weight (g)**	**T.N% in grain**
2.5 dSm^{-1}	3.39	5.50	35.56	1.48
4.0 dSm^{-1}	3.08	4.08	32.52	1.35
6.0 dSm^{-1}	2.65	3.25	29.22	1.07
L.S.D. 0.05	0.13**	0.99**	3.08**	0.14**
Sub main (Bacteria)				
Control	1.71	3.22	28.38	1.06
MSR-M49	3.48	4.44	33.08	1.34
MSR-H4	3.14	3.78	31.15	1.18

Mixed inoculum		3.83	5.67	37.12	1.62
L.S.D. 0.05		0.14**	0.54**	1.2**	0.11**
Interaction					
2.5 dSm^{-1}	Control	1.88	4.33	32.46	1.18
	MSR-M49	3.97	5.33	35.60	1.53
	MSR-H4	3.50	4.67	33.72	1.33
	Mixed inoculum	4.22	7.67	40.45	1.87
4.0 dSm^{-1}	Control	1.67	3.00	28.79	1.13
	MSR-M49	3.51	4.33	33.27	1.36
	MSR-H4	3.21	3.67	30.91	1.20
	Mixed inoculum	3.91	5.33	37.12	1.71
6.0 dSm^{-1}	Control	1.56	2.33	23.89	0.86
	MSR-M49	2.98	3.67	30.38	1.12
	MSR-H4	2.71	3.00	28.82	1.02
	Mixed inoculum	3.37	4.00	33.78	1.28
L.S.D. 0.05		0.23**	*n.s*	3.39**	0.19**

Spike number plant^{-1}), grain yield (g plant^{-1}), 1000-grain weight (g) and total N% in were decreased with increased soil salinity levels. Inoculation with MSR-H4 or MSR-M49 strains has a positive effect on these parameters. The duel inoculation with the selected microbes increased the previous parameters compared with other treatments at all soil salinity levels. At 6 dSm^{-1} soil salinity the grains yield was 3.37 g plant^{-1} with duel inoculation compared with 1.8 g plant^{-1} un-inoculated one.

3.4.2 Open field experiments

To evaluate the inoculation effect of the both microbes MSR-H4 or MSR-M49, field experiment was carried out during the period of (2018-2019) at Sakha Agricultural Research Station Farm with 5.2 dSm^{-1} soil. After maturation (130 days) different economical important parameter of wheat were collected this includes plant height, straw weight, spike number, Plant' spike weight, spike length Figure 2. A significant increase in all the parameter measured of MSR-M49, followed by MSR-H4. Interestingly dual inoculation of MSR-M49 and MSR-H4 have a larger impact of estimated parameters (Figure 2) suggesting a synergistic effect of the two strains for enhancing the growth of the wheat plant and saline soil.

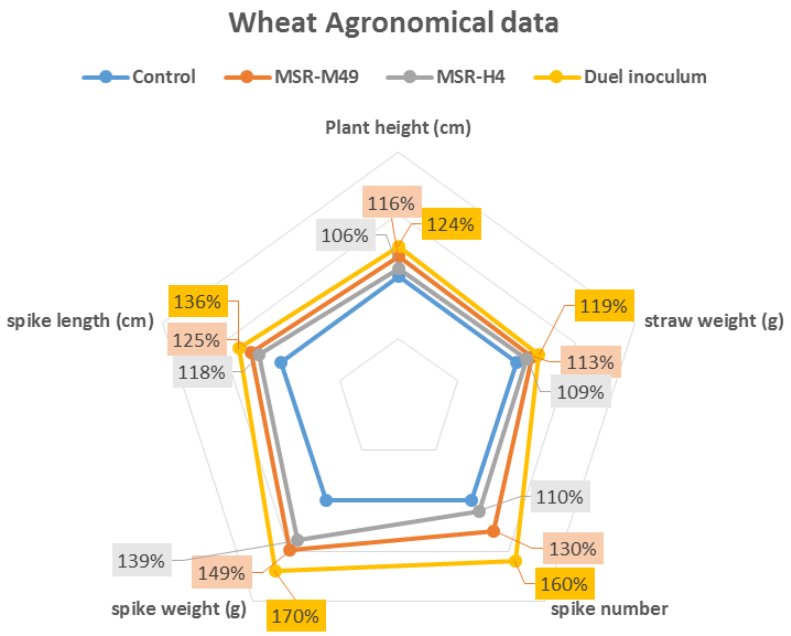

Figure 2: Different agronmical data collcted during the field trail Rader chart decribing the perecenage (%) increase in the treted plant in copmarison to control (un-incolulated). The innner line (blue) is representing the control with 100%, while the outer lines is repsenting single or duel incoulation. The concentration perecenage increase are presented for each parameter.

Yield content

1000 grain weighted and grain yield (ton fed^{-1}) of wheat in the field experiment was conducted in Table 5. Inoculation with PGPR and N_2-fixing strains MSR-M49 and MSR-H4 gave the greatest value compared with the un-inoculated ones. The duel inoculation with MSR-M49 and MSR-H4 were 35.230 g for 1000 grain dry weight and 2.86 ton field^{-1} compared with 29.097 and 2.50 at control treatment without inoculation, respectively.

Table 5: Yield and protein contents of the wheat grains

Treatments	1000 grain weight (g)	Grain yield (ton fed^{-1})	N% in grain	Protein in grain	Proline root (mg g^{-1} DW)	Proline shoot (mg g^{-1} DW)
Control	29.097	2.50	0.850	4.845	3.58	6.663
MSR-M49	31.550	2.77	1.213	6.916	2.79	7.727
MSR-H4	30.290	2.62	1.143	6.517	3.23	8.093
Duel inoculum	35.230	2.86	1.587	9.044	2.89	8.697
L.S.D 0.05	0.23**	0.06*	0.49*	1.83**	0.13**	0.03**

The chemical compositions of wheat grains (N% and protein), roots (K^+, Na^+ and proline) and shoots (proline) of treated plants were estimated in this study as showed in Table 5 and Figure 3.

The proline content in the roots of control was the highest value while the inoculation of MSR-M49 gave the lowest one (3.58 and 2.79 mg g^{-1} of dry weight, respectively). In the shoots proline content of duel inoculation was the greatest value while the control one was the lowest (8.69 and 6.66 mg g^{-1} of dry weight, respectively). The duel inoculation gave heights N% value compared with all treatments while control gave the lowest one (1.57% and 0.86%, respectively). Increasing in grains dry weight and N% in duel inoculation with PGPRs strains reflected in increasing protein content of the treatments at the same condition compared with all treatments including the single inoculation.

Na^+ and K^+ homeostasis

A variation of K-content of roots and shoots were obtained in this study as reported in table 6. Inoculation with *L. amnigena* increased K-content in roots while duel inoculation with MSR-M49 and MSR-H4 gave the highest shoots K-content compared with other treatments (35.850 and 79.033 mg/g of dry weight, respectively). Na-contents of roots and shoots were estimated and showed increased in Na-content in roots and shoots of control (18.487 and 12.617 mg g^{-1} of dry weight, respectively) compared with other treatments. The inoculation with *L. amnigena* effect on roots and shoots were the lowest value (15.373 and 10.143 mg g^{-1} of dry weight, respectively).

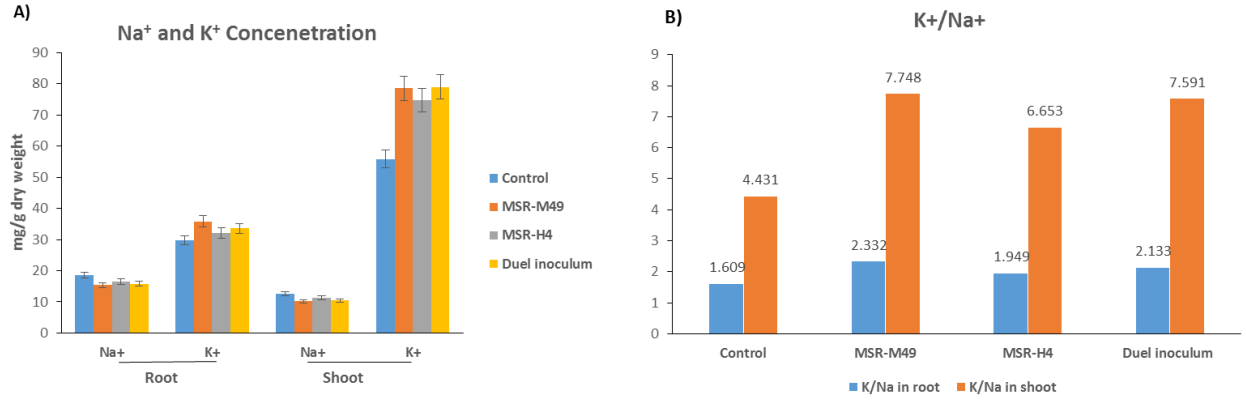

Figure 3: Na^+ and K^+ connotation in Wheat plants, A) Concentration mg g^{-1} plant material of Na^+ and K^+ in dry shoot and root B) The K^+/Na^+ ratio in wheat plant shoot and root inoculated with different bacteria strains.

Also, K^+/Na^+ ratio in root and shoots were estimated and reported in Figure 4b. The K^+/Na^+ ratio in the aerial part of the wheat also increased after bacterial inoculation.

The inoculation with MSR-M49 gave the highest K^+/Na^+ value of both roots and shoots with 2.332 and 7.748 ratios, respectively.

Discussion

Salinity stress is one of the more main abiotic stresses which results in significant harms in agricultural crop production, particularly in arid and semi-arid areas. Inoculation plant with growth promoting rhizobacteria (GPR) can help plant to grow in such stressful conditions and increased the productivity of crops **[4, 10].** Hence, in the present study, we explored the salinity stress alleviation of wheat by inoculation with salinity-tolerant PGPRs. *Lelliottia amnigena* belong to family Enterobacteriaceae The evolutionary history was inferred using the Neighbor-Joining method **[44]**. The optimal tree with the sum of branch length = 0.57904070 is shown. The tree is drawn to scale. The evolutionary distances were computed using the Maximum Composite Likelihood method **Tamura *et al.* [61]** and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. It worth mention that the classification of the Group B Enterobacteriaceae group is quite complex and using 16S rRNA alone could give indication of the taxonomical affiliation of the strains however more details analyses (whole genome sequence) will be required to identify the strains. Both strains MSR-M49 and MSR-H4 were able to produce. IAA as well solubilize the tricalcium phosphate. Interestingly, MSR-H4 **Furkan [62]** showed approximately 44% of the bacterial strains was found to have IAA production potential; e.g., *Bacillus* sp., Z. *halotolerans*, *Bacillus* sp., *B. gibsonii* , *O. oncorhynchi*, *Zhihengliuella* sp and *Halomonas* sp. IAA is produced after oxidation of indole-3-acetaldehyde by indole-3-acetaldehyde oxidase. Also, **Julie *et al.* [63]** reported that accumulation of IAA in the culture medium of wild-type *E. cloacae* UW5 occurred only in the presence of tryptophan. The MSR-H4 and MSR-M49 had the ability to produced polysaccharide and P-solubilizing which is highly important in promoting plant growth due to work as an active signal molecule during beneficial interactions. In our study chlorophyll content decreased with increasing salinity levels. Salinity induces osmotic stress and ionic toxicity that leads to secondary oxidative stress in plants **[64].** Inoculation with dual PGPR bacteria does as synergistic effect in Table 2. This approved with **Zhang *et al.* [65]** observed that *B. subtilis* GB03 increases the photosynthetic efficiency and chlorophyll content of *A. thaliana* through the modulation of endogenous signaling of glucose and abscisic acid sensing; thus the bacterium plays a regulatory role in the acquisition of energy by the

plant. In this context, **Dhanushkodi *et al*. [66], Rifat *et al*. [67]** and **Torbaghan *et al.* [68]** showed that inoculation with halotolerant bacteria improved the reduction of salinity effects on dry weight, plant height and production of wheat plants compared with un-inoculated treatments. Similarly, these dual traits bacterial strains were more effective than single trait strains under soil conditions (pot trial) in increasing root weight (up to 3.9-fold) and root elongation (up to 3.8-fold), dry shoot weight (up to 37.6%), number of tillers (up to 56%) an grain yield (up to 38.5%) as reported by **Baig *et al*., [69].** As a PGPB; *Bacillus* spp. strains significantly increased the dry shoot weight ranging from 30 to 160% over un-inoculated control **Baig *et al.* [69].** Previous studies have also been reported by other researchers that inoculation with P-solubilizing microorganisms improves growth and yield of wheat **[70]**. PGPB containing ACC-deaminase has also been reported to increase root growth in several plants (**71, 69**). **Mohite [72]** suggested that the IAA producing bacteria as efficient biofertilizer inoculants to promote plant growth and productivity. **Kotuby-Amazher *et al.* [10].** Evidenced that beneficial microorganism play a significant role in mitigate salt stress in wheat, performed an increased in crop yield and minified salt stress by approximately 50%. PGPR are nonpathogenic beneficial soil rhizobacteria play a key role in plant health and nutrition. These may benefit plant growth, either by improving plant nutrition or by producing plant growth regulators **[73]**.

PGPR can improve plant growth via biological nitrogen fixation, biosynthesis of phytohormones, nutrient solubilization, nutrient uptake, and host plant resistance to biotic and abiotic stresses **[74, 75].** In accordance with **Ozturk and Demir [76]** concluded that proline is known to occur widely in the higher plants and normally accumulates in large quantities in response to environmental stress. **Sheteawi and Tawfik [77]** Indicated that proline content generally increased in plants due to stress and the accumulation of proline may improve the cytoplasmic osmoregulation and thus, increase plant tolerance and biofertilized plants revealed higher values of these metabolic products than non-fertilized plants as response to their ameliorating and stimulating effect. Inoculation wheat with EPS producing *Bacillus insolitus* MAS17 and certain other *Bacillus* spp. enhanced the K^+/Na^+ ratio in plants by coating the root zones with soil sheaths **Ashraf *et al*. [27] and Han *et al*., [28].** Similar to our results which was visible in shoot and root moisture contents, **Soleimani *et al.*, [78]** and **Torbaghan *et al*., [68]** declared that the chosen bacteria amended Na^+ stress in wheat by increasing the relative humidity in plants and ion homeostasis. Bacterial

inoculation may diminished the inhibitory effect of salt stress on the roots and aid in the promote of more effective root systems, which could help plants absorb relatively more water from deeper soil under stress conditions **[79, 80].**

Conclusion

In general, the results showed that the isolated strains *B. halotolerans* MSR-H4 and *L amnigena* MSR-M49 have a great potential to improve wheat growth under saline soil. Based on our result we could concludes that both strains helped the plant to tolerate the salinity stress by decreasing the Na^+ ions toxicity and masking the effect of the salt. Dual inoculation increases in different agronomical parameter leading to increase of the wheat yield. In order to unravel the molecular mechanisms responsible of the PGPR activity, a genome sequence and transcriptomic analysis will be conducted on the two strains. The results demonstrated in this research provide a promising agricultural solution for increasing crop yields in semi-arid regions.

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