Employment of Halotolerant Plant Growth Promoting Bacteria to Mitigate Soil Salinity in Wheat and Chickpea

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Employment of Halotolerant Plant Growth Promoting Bacteria to Mitigate Soil Salinity in Wheat and Chickpea

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A PhD dissertation submitted in the partial fulfillment for the degree of Doctor of Philosophy (PhD) in Plant Sciences

By

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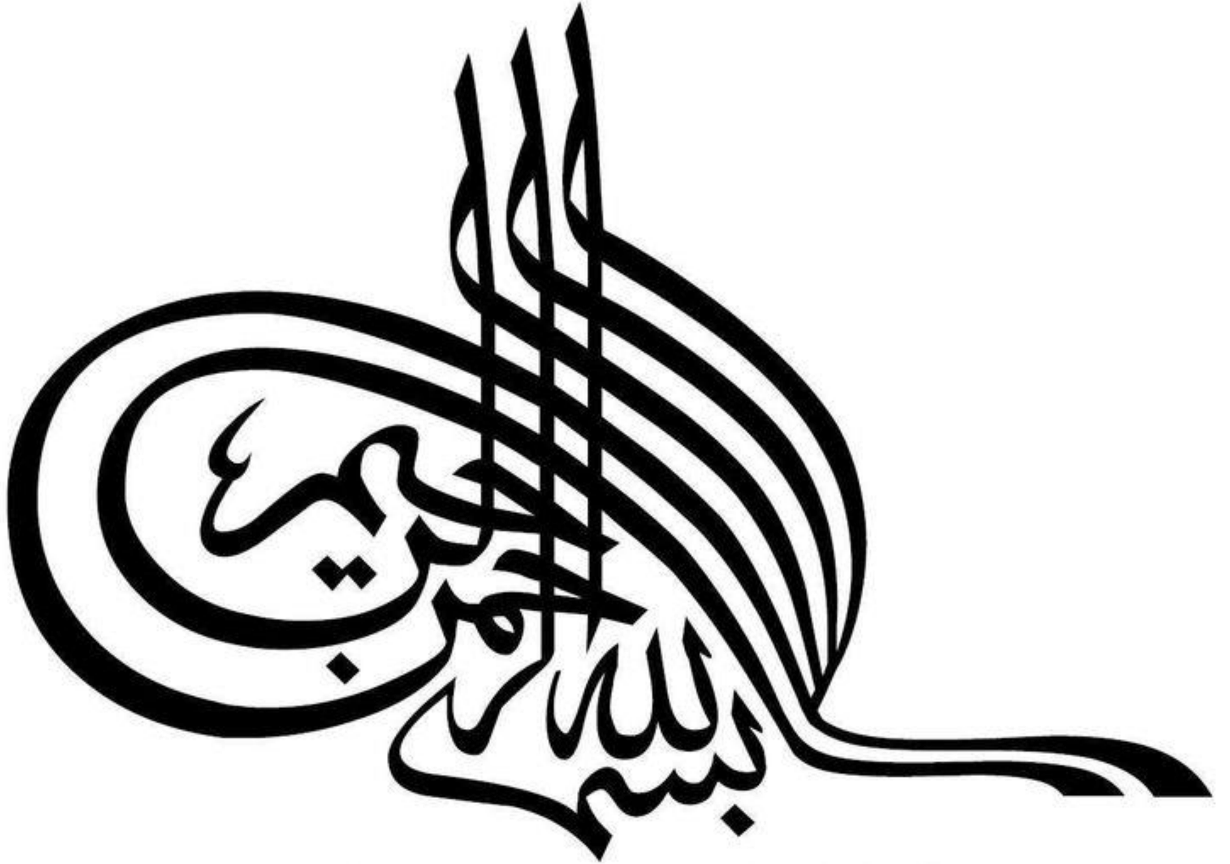

In the name of Allah,
the Most Beneficent,
the Most Merciful

In the name of Allah, the Entirely Merciful, the Especially Merciful.

All the praise is due to Allah, Lord of worlds.

The Entirely Merciful, the Especially Merciful,

Sovereign of the Day of Recompense.

It is You we workship and You we ask for help.

Guide us to the straight path. The path of those upon whom You have bestowed favour, not of those who have evoked Your anger or of those who are astray.

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Surah Al-Fatiha

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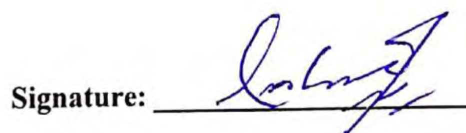
Signature: 

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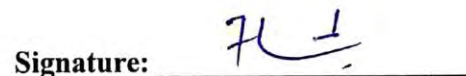
Signature: 

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DEDICATED TO

My Most Loving Parents

Mr. Haroon-ur-Rasheed & Mrs. Samina Rasheed

Who opened up avenues for me to learn and their loving support in every aspect enabled me to achieve my goals. My success is all yours. I love you both the most. I don't have strength to write late in front of your name Abu g. You are alive in my heart always.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
ACCD	1-aminocyclopropane-1-carboxylate deaminase
acdS	1-aminocyclopropane-1-carboxylate deaminase
ABA	Abscisic acid
ATP	Adenosine triphosphate
NH_4Cl	Ammounium chloride
ANOVA	Analysis of variance
ACE	Angiotensin 1-converting enzyme
APX	Ascorbate peroxidase
AsA	Ascorbic acid
AAS	Atomic absorption spectroscopy
BLAST	Basic local allignment search tool
BC	Before Christ
BNF	Biological nitrogen fixation
BGM	Botrytis grey mould
$CaCl_2$	Calcium chloride
$CaSO_4$	Calcium sulfate
CO_2	Carbon dioxide
CVD	Cardiovascular disease
CAT	Catalase
cm	Centimetres
CTAB	Cetyltrimethyl ammonium bromide
Cl^-	Chloride ion

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Chl	Chlorophyll
cDNA	Complementary deoxyribonucleic acid
CRD	Complete randomized design
$CuSO_4$	Copper sulfate
CK	Cytokinins
$dS\ m^{-1}$	Decisiemens per metre
°C	Degree Celsius
°E	Degrees east
°N	Degrees north
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DMSP	Dimethylsulfoiopropionate
K_2HPO_4	Dipotassium phosphate
Na_2HPO_4	Disodium phosphate
DW	Dry weight
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
Na_2EDTA	Ethylenedinitrilotetraacetic acid
EPS	Exopolysachharides
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferric sulfate
FAO	Food and agriculture organization
FTIR	Fourier transform infrared spectroscopy
FW	Fresh weight
GA3	Gibberellic acid

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GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutatione
GB	Glycine betaine
g	Gram
GDP	Gross domestic product
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
hr	Hour
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
Ci	Intracellular carbon dioxide concentration
LB	Lauria Bertani
LSD	Least significant difference
L	Litre
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MDA	Malondialdehyde
$MnSO_4$	Manganese sulfate
M	Metres
μ M/mg	Micro molar per milligram
μg/g	Microgram per gram

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ml	Microliter
µm	Micrometer
mg	Milligram
mg g^{-1} dw	Milligram per gram dry weight
mM	Millimolar
MMT	Million metric tons
ms/m	Millisiemens per metre
M	Molar
MEGA	Molecular evolutionary genetic analysis
ng	Nanogram
nm	Nanometer
NCBI	National center for biotechnology information
A	Net carbon dioxide assimilation
Pn	Net photosynthesis
NBT	Nitro blue tetrazolium chloride
NCCs	Nitrogen containing compounds
NPK	Nitrogen, phosphorus and potassium
N	Normal
OD	Optical density
ppm	Parts per million
%	Percentage
$HCLO_4$	Perchloric acid
POD	Peroxidase
PSI	Phosphate solubilization index
P	Phosphorus

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PGP	Plant growth promoting
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
PL	Pore length
PW	Pore width
K	Potassium
$K_2Cr_2O_7$	Potassium dichromate
K^+	Potassium ion
KH_2PO_4	Potassium phosphate monobasic
KCl	Potassium sulfate
PPD	p-phenyle diamine
PCA	Principal component analysis
pqqE	Pyrroloquinoline quinone
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
REL	Relative electrolytic leakage
RWC	Relative water content
RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SII	Salt injury index
SOS	Salt Overlay Sensitive

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SEM	Scanning electron microscopy
STI	Salt tolerance index
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaOCl	Sodium hypochloride
Na^+	Sodium ion
Na_2MoO_4	Sodium molybdate
Na_2SO_4	Sodium sulfate
g(s)	Stomatal conductance
SL	Stomatal length
SW	Stomatal width
H_2SO_4	Sulphuric acid
SOD	Superoxide dismutase
T	Temperature
TBA	Thiobarbituric acid
TSS	Total soluble sugar
TF	Transcription factor
tRNA	Transfer ribonucleic acid
Tr	Transpiration rate
TCA	Trichloroacetic acid
TSB	Tryptic soy broth
Trp	Tryptophan
TW	Turgid weight
US $	United state dollars
USSR	United socialist soviet republic

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VPD	Vapor pressure deficit
V	Volume
ZnO	Zinc oxide

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PUBLICATIONS FROM THESIS

S. No.	Title	Journal	Doi
1.	Halotolerant Plant Growth-Promoting Rhizobacteria Induce Salinity Tolerance in Wheat by Enhancing the Expression of SOS Genes	Journal of Plant Growth Regulation	https://doi.org/10.1007/s00344-021-10457-5
2.	Isolation of Halotolerant Bacteria from Rhizosphere of Khewra Salt Mine Halophytes and their Application to Induce Salt Tolerance in Wheat	Geomicrobiology Journal	https://doi.org/10.1080/01490451.2021.1946624
3.	Biofilm formation and flocculation potential analysis of halotolerant *Bacillus tequilensis* and its inoculation in soil to mitigate salinity stress of chickpea	Physiology and Molecular Biology of Plants	https://doi.org/10.1007/s12298-023-01280-1

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ABSTRACT

Soil salinity is one of the primary yield-limiting factors, in a variety of crops. Numerous rhizobacteria have shown an encouraging effect in improving the growth of plants and help them to cope with various stresses under various environmental challenges. This study was designed to isolate and identify halotolerant plant growth promoting bacteria (PGPB) from saline environment. These PGPB were further applied to the soil to mitigate salinity stress in wheat and chickpea.

In the first part of this study, twenty bacterial strains were isolated and identified from the rhizospheric soil of *Justicia adhatoda*, *Chenopodium murale*, and *Cenchrus ciliaris,* growing in Khewra salt mine. It is the world's second-largest salt mine. Six of these bacterial strains were discovered to be extremely salt tolerant, as they could grow in Luria Bertani (LB) medium, supplemented with 10% NaCl. On the basis of their morphological, biochemical, and partial 16S rRNA gene sequencing, isolated bacteria were characterized and identified as *Bacillus megaterium*, *Bacillus tequilensis*, *Bacillus xiamenensis*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, and *Staphylococcus pasteuri. B. megaterium*, *B. tequilensis*, and *P. putida* were chosen for further examination based on the best plant growth-promoting activities and extracellular enzyme secretions. Selected PGPB demonstrated varying degrees of antibiotic tolerance. Among three selected bacterial strains, *B. tequilensis* exhibited tolerance against maximum number of antibiotics. All of the chosen bacterial strains were capable of producing phytohormones such as indole-3-acetic acid (IAA), gibberellic acid (GA3), and abscisic acid (ABA). Under salt stress conditions. These bacterial strains increased root length, shoot length, and leaf area of wheat seedlings by increasing macronutrient uptake (nitrogen, phosphorus and potassium).

In the second part of study, selected plant growth promoting rhizobacteria (PGPR) including Plant growth promoting attributes of *B. megaterium*, *B. tequilensis*, and *P. putida* were investigated further. These strains exhibited potential to secrete 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and exopolysaccharides (EPS). These bacterial strains improved the physiology, biochemistry, and antioxidant enzyme activities of the wheat plant under salt stress. Plants inoculated with PGPR exhibited higher relative water content, higher photosynthetic pigments, lower levels of hydrogen peroxide

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(H_2O_2) and malondialdehyde (MDA), and improved enzymatic activity for the scavenging of reactive oxygen species (ROS). The qPCR revealed increased expression of salt overly sensitive genes (SOS1 and SOS4), indicating their possible role in stress tolerance. These genes can be overexpressed in wheat plants to make them more resistant to salinity stress. Based on these findings, it is possible to conclude that priming seeds with the aforementioned PGPRs can reduce the negative effects of salinity on wheat plants.

In the third part of the study, salinity tolerance mechanism of one best performing halotolerant bacterium (*B. tequilensis*) was investigated. Scanning electron microscopy (SEM) revealed the highest floc yield and biofilm formation ability of *B. tequilensis* at 100 mM NaCl concentration. Fourier Transformed Infrared Spectroscopy (FTIR) depicted the presence of amino, hydroxyl and carboxyl groups in the exopolysaccharides (EPS) of *B. tequilensis*, indicating the presence of carbohydrates and proteins. Using PCR, plant growth promoting bacterial genes viz., 1-aminocyclopropane-1-carboxylate deaminase (acdS) and pyrroloquinoline quinone (pqqE) were successfully amplified from the genome of *B. tequilensis*. To further confirm its plant growth promoting ability, *B. tequilensis* was inoculated in the soil and chickpea plants were grown. Chickpea seedlings displayed increased chlorophyll content, relative water contents, higher soluble sugars, and proline contents, while their electrolytic leakage was decreased. The activity of several antioxidant enzymes, including peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), was increased. Salt stress also resulted in higher production of malondialdehyde and hydrogen peroxide (H_2O_2).

In the fourth part of the study, *B. tequilensis* (strain MPP8) was inoculated to chickpea plants at 100 mM NaCl concentration and the expression of CaRab genes was investigated, using qRT-PCR. These genes have been reported to be involved in intracellular trafficking and play key role in salinity stress. In this study, the expression of CaRabA2 gene was found to be higher than all other genes. A strong positive correlation (R2 = 0.6615) of CaRabA2 gene expression with Na^+ buildup in leaves was observed. Moreover, a variety of leaf traits like stomata assay, gas exchange assay, and the concentrations of sodium (Na^+) and potassium ions (K^+) in the leaves were also studied. Under salinity stress, net CO_2 absorption, intracellular CO_2 concentration, stomatal conductance, net transpiration, and photosynthetic rate were observed to be decreased.

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Saline conditions increased leaf vapor pressure deficit and leaf temperature. Inoculation of *B. tequilensis* improved gas exchange properties of chickpea and improved plant vigor. At 100 mM NaCl stress conditions, limited gaseous exchange was observed due to closed stomata and deformed guard cells. Findings of this study suggested the sustainable use of *B. tequilensis* to mitigate salinity stress of chickpea and other crops.

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1. INTRODUCTION

1.1 WHEAT (*Triticum aestivum*)

Triticum aestivum is a species of Poaceae family and it is the third most important cereal crop of the world. It is grown in winter, spring, and facultative seasons. Wheat was initially planted around 11000 years ago, and as geological farming expanded, bread wheat became the most important food supply from China to England (Dubcovsky and Dvorak, 2007). It is the mostly grown and consumed crop for the nourishment of human and animals. It is the principal staple food of about 36% of the total world population. Wheat is the major Rabi (winter) crop in Pakistan. Among major cereals grown in Pakistan, wheat ranks first, followed by rice and its local names in Pakistan are Kanak and Gandum. In Pakistan, nearly 30 different wheat cultivars are grown, 22 of which are reported to be particularly efficient under local conditions (Rattu, 2011).

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1.2 ECONOMIC IMPORTANCE OF WHEAT IN PAKISTAN

Wheat is one of Pakistan's most important cereal crops, and its future food security is dependent on it. Pakistan is the 10th largest wheat producer in the world, accounting for 3% of worldwide wheat production. In Pakistan, wheat is the most common food grain. It implements a variety of horticulture policies. This increases the value of horticulture by 14.4% and the gross domestic product by 3.1% (Afzal *et al*., 2015). Wheat production was expected to reach 25.7 million metric tons (MMT) in 2020-2021, over 6% more than the revised agricultural output of 24.3 MMT, the previous year. Pakistan's wheat exports for MY 2019/20 was expected to be approximately 600,000 metric tons. The government increased the wheat upkeep cost for the MY 2020/21 harvest to Rs. 1400 per 40 kilograms ($226 per metric ton) from Rs. 1300 per 40 kilograms ($210 per metric ton), previous year. Pakistan's wheat trades for MY 2019/20 were reached over 600,000 tons. Wheat is largely shipped to Sri Lanka, Dhaka, Gulf countries, Afghanistan, and a few African countries. The government halted wheat shipments in October 2019 due to depleted stockpiles and rising expenses, and Pakistan has not shipped wheat since then. Pakistan's government announced that 300,000 tons of duty-free wheat imports will be allowed until March 2020.

Pakistan's domestic wheat sector is supported by a guaranteed wheat price of $226 per metric ton. The government only captures roughly a fourth of the harvest (half is left in towns and a quarter goes straight to the "open" market). The acquisition cost adequately establishes the market price of wheat in Pakistan. The levy is significantly below Pakistan's 150% bound tax rate (the most extreme tax rate Pakistan can impose) on wheat (Source: Global Agricultural Information Network Grain and Feed Annual Report 2020) (https://www.fas.usda.gov/data/pakistan-grain-and-feed-annual-3).

1.3 MEDICINAL IMPORTANCE OF WHEAT

Wheat contains several medicinal qualities. Red and white wheat and durum wheat grains possess the antioxidant activity and phytochemical content. Wheat is used to treat various ailments. Some of them are enlisted below:

1.3.1 Treatment of Disorders of Tooth

Wheat is useful in the treatment of inflammation of gums and tooth sockets. Wheat grass juice is considered as a magnificent mouth wash for the treatment of sore throats and inflammation of gums and tooth sockets, also anticipates tooth rot and tooth pain. When you chew the wheat grass, it draws out poisons from the gums and in this way checks bacterial development (Kumar *et al*., 2011).

1.3.2 Treatment of Constipation

Wheat grain is wasted in milling of the flour. It is more nutritious and healthful than the wheat flour. It is an excellent purgative. The grain of wheat is used for the prevention and treatment of constipation and helps in easy evacuation (Kumar *et al*., 2011).

1.3.3 Treatment of Skin Diseases

Some bacteria cause skin infection and ulcerated wounds that can be treated with wheat grass, which promotes cell activity and normal growth. Wheat grass juice creates an unfavorable environment for the growth of bacteria. Plaster of wheat grass juice is utilized as a sterilizer and can be applied on tainted zone. Wheat flour is helpful as a tidying powder over swollen surface as in blisters and burns. To expel spots, entire wheat flour is blended

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with vinegar and afterward bubbled. This blend is applied apparently to evacuate spots (Kumar *et al*., 2011).

1.3.4 Treatment of Digestive System Disorders

Enema is the injection of some liquid to get relief from disorders of the colon, constipation, colitis and bleeding piles (Hvatum *et al*., 2006). Wheat grass juice is used as an anemia (Kumar *et al*., 2011).

1.3.5 Treatment of Circulatory Disorders

Functions of heart and lungs can be improved by chlorophyll content present in wheat. Lungs function better if iron content increases in blood and hemoglobin. It lessens the effect of CO_2 and improves oxygenation. Wheat grass juice is suggested for circulatory system disorders (Jacobs *et al*., 1998).

1.3.6 Treatment of Scars

Wheat is baked on fire until it turns black and then it is ground to make a fine paste. This paste is put in a thin cloth and compressed to get oil. This oil can be applied on the scars regularly, for their treatment (Kumar *et al*., 2011).

1.3.7 Treatment of Chest Pain

Mixture of wheat, barn and coarse salt has been reported to be very useful for the relief of chest pain (Kumar *et al*., 2011).

1.3.8 Treatment of Tonsils Pain

To get rid of tonsils pain, dumplings are prepared by mixing water in wheat flour. This material is placed in cloth and incited on tonsils (Kumar *et al*., 2011).

1.3.9 Treatment of Pimples

A fine paste of whole wheat is used for the treatment of acne (Kumar *et al*., 2011).

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1.3.10 Treatment of Colon Cancer

Wheat bran reduces colon carcinogenesis, and it is more useful than oat grain or con grain (Reddy *et al*., 2000).

1.4 NUTRITIONAL IMPORTANCE OF WHEAT

Wheat is the source of 55% of carbohydrates, 20% of nourishment calories, significant phytochemicals, minerals, nutrients, amino acids, and dietary fiber segments in human feed. Gluten substance and low amylase are two important elements or characteristics that help wheat flour to combine with various flours, such as grain and pulses. The presence of gluten protein gives viscoelastic properties to wheat to prepare bread, pasta, noodles and other food items (Breiman and Graur, 1995; Shewry, 2009).

1.5 ENVIRONMENTAL STRESSES IN WHEAT

Various environmental stresses have negative effects on crop growth and its yield. Abiotic stresses include salinity, drought, salinity, radiations, temperature fluctuations, floods, heavy metals and pollutants (both inorganic and organic) and they limit crop productivity (Lawlor and Cornic, 2005). The wheat gene pool contains enough genetic diversity to deal with these challenges (Trethowan and Mujeeb-Kazi, 2008; Lichtenthaler, 1996). Major biotic and abiotic yield limiting factors have been described in Fig. 1.1.

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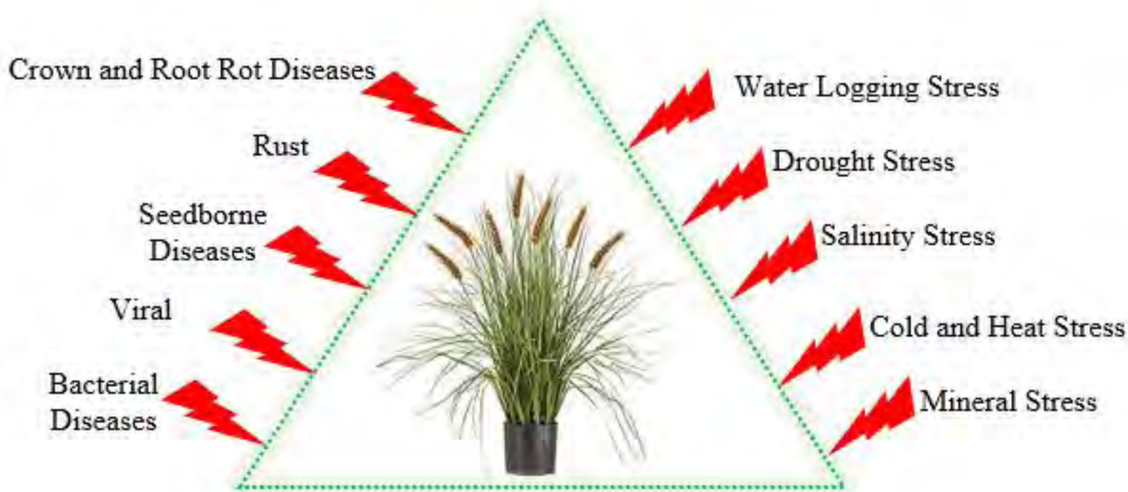

Fig. 1.1 General stresses in wheat (Afzal *et al*., 2015).

1.5.1 Biotic Stresses

Wheat crop is affected by various insects, fungal, bacterial, and viral diseases (McIntosh, 1998). There are two main classes of plant diseases:

i. Parasitic diseases
ii. Nonparasitic diseases.

Parasitic disorders are caused by living beings, for example, microbes etc. while nonparasitic diseases are chiefly brought about by mutagens and ecological variables. Wheat is mostly influenced by parasitic species. These diseases mainly include rust of leaf, stem, and stripe. In wheat, various diseases are categorized as seedborne, viral and rusts (Afzal *et al*., 2015). Seedborne diseases contaminate seeds from parasitic worms, insects, or soil. These diseases cause loss of vigor and result in seedling blight and it becomes toxic to human beings. Some of the most common seedborne diseases of wheat include leaf spot diseases, kernel bunt, leaf blotch, head blight, loose smut, head blight or scab, common bunt, root rot and crown diseases (Majumder *et al*., 2013).

Root rot and root crown diseases are caused by soilborne fungi. The infected root and shoot of the young seedlings appear dry and rough, while in some cases, alternatively yellow and brown stripes appear on whole length of the leaf blades. After one year, when wheat is planted in the same field, these diseases occur more frequently. The disease severity increases if grass weeds grow in the wheat field. Root and crown rot diseases in oat, barley and wheat are not very common but are damaging diseases, that are caused by various soilborne and seedborne fungi. Root rot caused by *Cercosporella herpotrichoides* is common in winter wheat fields whereas root rot caused by *Fusarium* and *Helminthosporium* are widespread in spring wheat areas. Viruses that cause diseases in wheat crop are vectored by mites, planthoppers, beetles, aphids, leafhoppers, seeds, pollens and the root-inhabiting fungus *Polymyxa graminis* L. Wheat yellow dwarf and wheat yellow mosaic are the most common viral diseases of wheat (Jones and Sutton, 1996).

Wheat plant has been reported to be infected by eight different rust fungi. Famous types of rusts include stem rust (caused by *Puccinia graminis* f. sp. tritici), stripe rust (caused by *P. striiformis* f. sp. tritici), black leaf rust (caused by *P. triticina)*, brown rust

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(caused by *P. recondita*) and yellow rust (caused by *P. striiformis)* (Cummins, 2013). Certain rusts come in a variety of breeds and are only found on specific hosts. These rusts produce urediniospores and aeciospores to cause diseases in grain and grasses. About 350,000 spores are formed by one rust pustule and disperse quickly to infect host plant (Peterson, 1974). Rust fungus develops its spores on the leaf and stem of wheat plant for ultimate crop damage. The rust pathogen uses water and important nutrients that are required for the growth and development of wheat kernels. So, kernels are shrunken to such a remarkable level that several of them become underweight and blow away. The other kernels shrink to half or a third of their original size (Schumann and D'Arcy, 2010).

1.5.2 Abiotic Stresses

Soil and water supplies per unit of population are steadily decreasing and will decline over the next decade. Yields must be increased by up to 1.6 % per annum to satisfy the rising global demand for wheat. To deal with the dilemma of maintaining the population growth and alleviating global poverty, as well as increasing crop productivity in an environment that fosters sustainable intensification, several solutions must be identified. Sustainable farming can also contribute to the solution of this problem by developing stress resistant and high-yielding cultivars that can increase output by up to 50% (Lynam, 2004). Abiotic stresses are main growth restricting variables in wheat. The main goal of scientists in the 21st century is to enhance and ease crop production in areas where the climate is highly unpredictable and unstable, which is one of the greatest roadblocks to farming systems (Mahajan and Tuteja, 2005). Agricultural maintenance, as well as tolerance to a variety of environmental stresses, flexibility, and production economy, will ultimately determine productivity in stressed circumstances (Brown and Rieseberg, 2006). As a result, research should focus on enhancing our information and description of target climate factors, implementing innovative selection, and screening procedures, selecting adaptive morphophysiological stress features, and implementing the most sustainable agricultural management tests (Afzal *et al*., 2015).

High temperature is impacting the production and yield of wheat crop. Heat stress in wheat is a global issue. On exposure to heat stress, wheat plants show a variety of

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morphophysiological changes. Over the last decade, heat stress has resulted in major reductions in wheat yields, with the world struggling to meet record production levels, posing a critical threat to food safety. Spring wheat is typically more delicate than winter wheat. The percentage of germination can be significantly reduced by heat stress exceeding 30 °C, which can double the time of germination. This also affects the development of roots, which decreases the usual amount, dramatically. Elevated temperatures, usually exceeding 34 °C, result in the reduction of the duration of grain filling which ultimately affects final grain weight due to suppression of photosynthesis (Brestic *et al*., 2014) and immediately constrain the production of endosperm (Telfer *et al*., 2013).

Wheat can survive in a low temperature range of 1-4 °C, a lowest possible growth temperature as the most adaptable crop. Plant reactions to sub-zero conditions are generally referred to as cold stress (Gusta and Chen, 1987). Cold stress affects around 80 million hectares of total agricultural growing area worldwide. As nighttime, temperatures drop below 10 °C in equatorial regions and spring wheat is more disturbed at low temperature and the growth of shoot and root is affected. Cold environments can delay flowering period of plant or cause severe infertility prior to flowering (Sanghera *et al*., 2011). Low temperatures dramatically reduce root growth in the case of winter wheat, with increased concentration of sugars and a remarkable reduction in water potential. Typically, leaves of winter wheat can preserve more water and are relatively smaller in size, as compared to spring ones. The protein, sugar and lipid contents accumulation double up in winter wheat to withstand even lower sub-zero temperatures (Fowler, 2001). The acclimatization and cold stabilization mechanisms boosts up survival under very low temperatures. During cold acclimatization, substantial increase in glutathione, proline, dehydrins and TaADF levels, which perform a crucial part in dropping the osmotic potential, is observed (Abdin *et al*., 2002).

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Drought is another major constraint to influence the development, growth, and production of wheat, worldwide. Drought condition affects about 45% of wheat production (Fedoroff *et al*., 2010). The growth of plant is stunted under the influence of drought and the development of roots is also inhibited. Limited water resources are the main factor of

drought conditions in Pakistan. Canal water is just used to irrigate 6.34 million hectares of cultivated land out of the 22.45 million hectares while 12.52 million hectares are irrigated by tube wells and new resources. For the remaining 3.59 million hectares, there is no water available (Khalil *et al*., 2014).

The common occurrence of waterlogging stress is observed in more than 10 million hectares, worldwide, in both elevated rainfall and irrigated environments. In susceptible cultivars, there is a massive decrease in root and shoot mass, with a substantial decrease in gas exchange through stomata. In roots, the mineral concentration increases, while it decreases in shoots. Cultivars with efficient parenchymatic tissues for transport are recognized to be resistant in drenched environments. There are substantial variations in genetic diversity for resistance among varieties, however their frequency is lesser. However, the distribution is additive and simple (Barrett-Lennard *et al*., 1988).

Mineral stress is another key restraint to plant growth and it is described as a lack of vital nutrients or toxicity of nutrient or non-nutrient minerals (particularly Al, Na, Cl, Mn, and other heavy metals) throughout the earth's land surface (Lynch and Clair, 2004). Globally, approximately 40 million ha of wheat cultivated fields come under the influence of mineral stress, primarily due to soil acidity and basicity. Inorganic fertilizers alteration, drainage of effluent and wastewater and industrial discharges are accountable for mineral stress (Ranieri *et al*., 2005).

The term salt-affected applies to saline or sodium soil (Szabolcs, 1989). In the saline soil, there is an excess of soluble salt, a liquid found within soil aggregates. In the sodic soil, sodium is usually associated with clay particles. Salt stress occurs when anthropogenic or environmental activities are assumed to contribute to the increase of salts in the groundwater that restricts the plant development and growth. Normal salinity is caused by the erosion of rock, which creates dissolved salts of numerous types, mostly sodium chlorides, sulphates, and carbonates, as well as magnesium and calcium to a lesser extent. Sodium chloride is soluble to a greater extent. The deposit of sea salt in the atmosphere also contributes to salinity. Salinity resulted from anthropogenic activities

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alters the soil's hydrological equilibrium among the crop water (transpiration) and water (irrigation or precipitation). The extremely widespread reasons are:

i. Renewal of land and the replacement of agricultural crops with perennial vegetation.
ii. Irrigation systems that use salt-abundant water to irrigate crops or have inadequate drainage (Munns and Tester, 2008).

Salt stress in soil also adversely affects the productivity of wheat. The wheat yield begins to decrease at 6-8 dS m^{-1} (Royo and Abió, 2003). Ionic stresses arise under salt stress because low soil water capacity and surplus accumulation of Na^{+} inside the plant. It is also related to imbalance of nutrients (Huang *et al*., 2010). Reduced development and growth, altered reproductive performance and reduced yield is observed in the plants that are under the effect of salinity (Turan *et al*., 2009). In plants, the overall growth is affected under salinity due to oxidative pressure, changes in the enzymatic activities, disturbance in photosynthesis, changes in structure and function of bio-membranes, injury to organelles, and imbalance in hormone (Çelik and Atak, 2012; Hasanuzzaman *et al*., 2014a; Hasanuzzaman *et al*., 2014b).

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Salinity is the utmost destructive abiotic stresses with tremendous adverse consequences including water uptake efficiently via xylem, uptake of nutrients, photosynthetic rate, enzymatic activities, and disturbances in morphophysiological, biological and chemical features of plants. Various researchers have demonstrated the impact of salinity on various salinity resistant and vulnerable cultivars of wheat. In wheat, increase in salt concentration marks the decline in the photosynthesis and transpiration and increase in the uptake of sodium and chloride ions that disrupts the usual metabolism (Fig. 1.2).

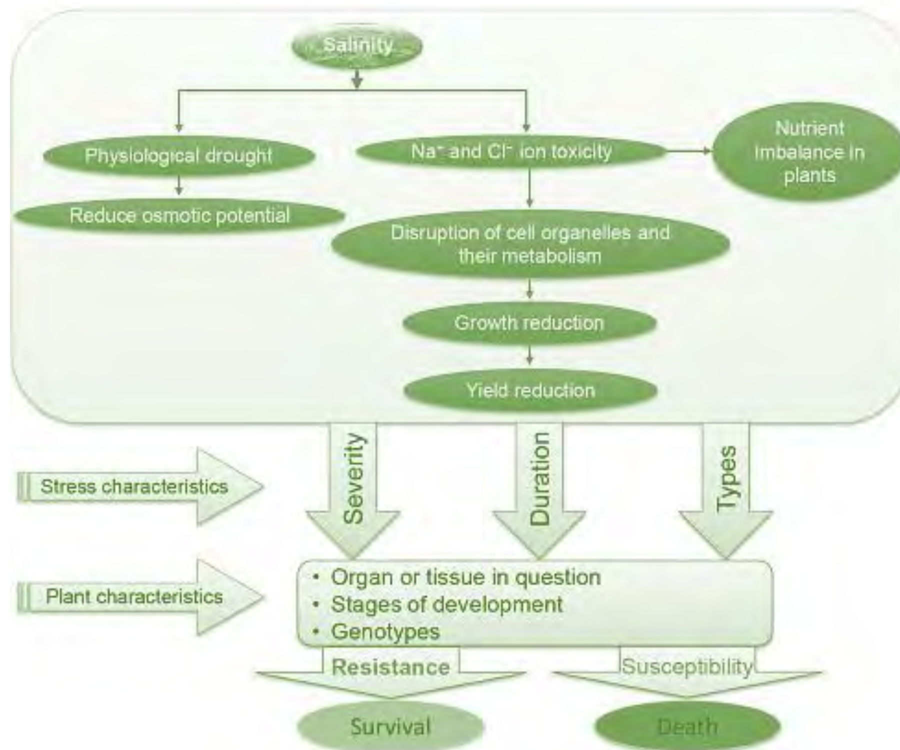

Fig. 1.2 General scheme for reactions to salt stress and adaptation in plants (Hasanuzzaman *et al.,* 2017).

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1.6 CHICKPEA

Chickpea (*Cicer arietinum* L.) belongs to Fabaceae family. It is an earliest world pulse and it has been reported to be primarily cultivated in Central and West Asia, Ethiopia, South Europe and North Africa (Ladizinsky and Adler, 1975). Chickpea has been originated in the region of modern south-eastern Turkey and adjacent Syria. Wild varieties of chickpea have been reported in these areas, particularly *Cicer reticulatum,* which shared common ancestry with chickpea and can be considered as its wild progenitor (Van Der Maesen, 1987). The authentic documentation for the cultivation of domestic chickpea dates to 3300 BC in Egypt and Middle East, due to scarcity of its archeological records (Van Der Maesen and Maxted, 2007). Origin of chickpea was also tracked by De Candolle (1883) to southern regions of the Caucasus and northern areas of Persia. Two primary centers of chickpea diversity were categorized by Vavilov (1926) in East Asia and the Mediterranean, and a secondary one, Ethiopia. Seed size was the basis of this classification and it was

elucidated that small-seeded cultivars of chickpea were predominately distributed in Eastern zones and large-seeded cultivars abounded around the Mediterranean basin (Vavilov, 1926). About two centuries ago, cream-coloured and large-seeded chickpea might reach India through Kabul, Afghanistan, hence named as Kabuli chana. Dark-coloured and small seeded chickpea are called Desi or Local cultivar (Van Der Maesen, 1972). Genetic evidence and karyotypic analysis also illustrated that *Cicer arietinum* is decedent from its wild cultivar *Cicer reticulatum* (Ohri and Pal, 1991).

The word Cicer is of Latin origin, meaning force and strength while the word arietinum refers to the morphology of the seed, which resembles the head of a ram. (Singh and Diwakar, 1995). Cicer belongs to tribe Cicereae Alef. (Kupicha, 1977). There are 43 described species: 9 annual (including the cultivated one), 33 perennial, and 1 unspecified (Singh, 1997; Coles *et al.,* 1998). Van der Maesen (1972) concluded that *Cicer arietinum* L. is the most ancient and extensively used grain crop in the Middle and Far East and selection and mutation resulted in the evolution of chickpea (Kupicha, 1977).

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1.7 BOTANICAL DESCRIPTION OF CHICKPEA

Chickpea is an annual plant, herbaceous in nature with bushy appearance, 0.2 m tall and dark green or bluish green in color. It is mostly covered with hairs, either glandular or non-glandular and has diffused branching system (Singh and Diwakar, 1995). These hairs produce acidic exudates to confer resistance against insect pests (Rasool *et al.,* 2005). Chickpea seedlings are hypogeal in nature. Vertical shoot emerges from the plumule. Two to three pairs of leaflets along with a terminal one, emerges from initial true leaf. Leaves are arranged alternatively at each node and they are unipennate compound (Singh, 1990). Leaves have 11 to 13 leaflets arranged on a rachis with a small petiole (Rasool *et al.,* 2005). Plant has a complex root system, deeper than 120 cm (Sheldrake and Saxena, 1979). This enables chickpea to cope with low water availability and enhances its adaptability towards cooler regions, having low rainfall (Rasool *et al.,* 2005). The stem of chickpea is divided into primary, secondary and tertiary branches which are generally quadrangular, grooved, and green. The stature of chickpea stem is upright, bifurcated, glutinous, hairy, terete, herbaceous, solid and green (Singh and Diwakar, 1995).

Flowers are arranged singly at the axillary position or form inflorescence of two or three flowers. Variety of flower colors appears in chickpea like pink, white, blue or purple. Among desi and kabuli types, the difference lies in corolla color; it is white in kabuli and purple in desi chickpea. Chickpea flower has one carpel and diadelphous stamens which mean filaments are fused in nine stamens and free in tenth one. Mostly self-pollination occurs but cross pollination also takes place. After the extension of filament, pollens fall from anther to pistil. Both the anther and the pistil reside inside the keel (Singh and Ibrahim, 1990). In chickpea, pod development occurs acropetally (Sheldrake and Saxena, 1979). Pod size in chickpea fluctuates significantly and environment has little effect on this character. Pod number ranges from few to 1000 pods per plant in chickpea and appear to be inflated (Singh, 1990). Chickpea pod is a rhomboid ellipsoid, with typically 1-3 seeds (Rasool *et al.,* 2005). Pod-filling varies from 8.97 to 56.53% and its fluctuation has significant dependence on weather (Pundir *et al.,* 1992). Chickpea is a rabi crop that is often grown on sandy loam soil and has a maturity time of 95-110 days (Rasool *et al.,* 2005).

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1.8 DISTRIBUTION OF CHICKPEA

The cultivated chickpea (*Cicer arietinum* L.) is the 5th most vital leguminous crop after soybeans (*Glycine max*), groundnuts (*Arachis hypogaea*), dry beans (*Phaseolus vulgaris*), and peas (*Pisum sativum*) (Ohri and Pal, 1991). Chickpea belongs to family Fabaceae and subfamily papilionoideae. Initially, it was positioned in tribe Vicieae but later it was placed in tribe Cicereae (Kupicha, 1977). The literature studies indicate the dispersal of chickpea in different areas but prehistoric record of cicer distribution can only be surmised. It was proposed that the spreading of chickpea was done by the Western Aryans (the Pelasgians and the Hellenes) westwards to the Mediterranean and eastwards to India (Van der Maesen, 1972). The two varieties of chickpea are geographically distributed in different regions. The kabuli is circumscribed in Western Mediterranean while that of desi variety occurs in eastern Mediterranean to central Asia and sub-continent (Moreno and Cubero, 1978). Selection for better quality and color led to the evolution of kabuli variety from desi one (Berger and Turner, 2007).

Following the termination of the last ice Age, chickpea was one of the earliest crops that invaded Europe (Mikic, 2013). In India, chickpea is thought to be recently introduced, about 1700 BC, specially the kabuli variety (Berger and Turner, 2007). As chickpea is cosmopolitan, different indigenous or vernacular nomenclatures are used for its identification such as Hamas (Arab nations), Kichererbse (Germany), Ceci (Switzerland), Lablabi (Turkey), Garbanzo (Spain), Simbra (Ethiopia) and Chana or Gram in subcontinent (Singh *et al.,* 2014).

1.9 CHICKPEA PRODUCTION

Globally, the annual production of chickpea is 10.1 million tons and thus graded 3rd among pulse crops (Muehlbauer and Sarker, 2017). Currently, more than 40 countries are cultivating chickpea worldwide and Asia accounts for 91.8% and 90.4% of global area and production, respectively (Ahlawat, 2012). The area specified for production of chickpea is 89.7% in Asia, 4.3% in Africa, 2.6% in Oceania, 2.9% in the Americas and 0.4% in Europe (Girma *et al.,* 2017). In Asia, the total area of cultivation for chickpea is 10.049 m ha and its productivity is 773 kg/ha (Ahlawat, 2012). Based on period, species, cultivar and management applications, average grain yield in South-Asia ranges from 300 to 14,000 kg ha^{-1} (Chibarabada *et al.,* 2017). The chief producers of chickpea are India, Turkey, Pakistan, Mexico, Ethiopia, Australia and Spain (Ahlawat, 2012). India, being a leading chickpea producing country, accounts for 70% of overall world chickpea production (McVay and Crutcher, 2016; Muehlbauer and Sarker, 2017).

In sustainable agriculture, desi cultivar accounts for more than 80% of whole chickpea production (Saxena and Singh, 1987). After India, the next chief producers are Pakistan and Iran and produce 10% and 5% of world production, respectively (Muehlbauer and Sarker, 2017). In Pakistan, chickpea agriculture covers 1028.90 thousand hectares, yielding 479.5 thousand tons with an average yield of 466 kilograms per hectare (Shah *et al.,* 2007). Punjab's Khushab district has the greatest production (28%) of chickpea (Govt. of Punjab, 2005). Cultivation of chickpea is done for different purposes like food, conservation of environment, nutrition and other economical purposes (Girma *et al.,* 2017).

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1.10 MEDICINAL IMPORTANCE OF CHICKPEA

In addition of providing protein and fiber, chickpea is considered as "functional food" which means it comprises of physiologically active compounds, which are helpful in combating different diseases (Hasler, 2002) and modified constituents that provide unique benefits to human health (Milner, 2000; Jukanti *et al.,* 2012). High content of flavonoids and polyphenols offers antioxidant properties to chickpea (Rachwa-Rosiak *et al.,* 2015). In chickpea seed, the total anthocyanin content is 14,9 mg/kg of bean while that of polyphenols is reported as 0.72 to 1.81 mg/g of bean (Segev *et al.,* 2010). Chickpea consumption decreases the total serum cholesterol and low-density lipoprotein-cholesterol (LDL-C), thus reducing the risk of coronary heart disease (Gupta *et al.,* 2016). Chickpea is also considered as an appropriate diet for diabetic patients because it releases glucose in blood in a slow speed. Starch in chickpea is extensively polymerized, and this renders it more resilient to digestion in intestinal region, resulting in slower entrance in bloodstream and decreasing insulin requirement (Gupta *et al.,* 2016).

Kumar *et al*. (2014) elucidated the mechanism of biologically active components in chickpea, exhibiting anti-cancerous activity against human oral cancerous cells. Moreover, colon cancer is also suppressed by several bioactive compounds (Murillo *et al.,* 2004). One of the main causes of cardiovascular diseases (CVD) and stroke is hypertension. Chickpea proteins are found to have inhibitory effect against hypertension activating enzymes that are angiotensin I-converting enzyme (ACE) (Pedroche *et al.,* 2002; Barbana and Boye, 2010; Mark and Davis, 2000). Worldwide, the major apprehension of people is obesity because it increases the risk of health issues. Chickpea, being a rich source of dietary fiber like other lentils, helps to tackle the problem of obesity (Blackwood *et al.,* 2000). Isoflavones in chickpea are effective against osteoporosis (a silent ailment of aging) as well as exhibit estrogenic properties, hence heralded as phytoestrogens (Fahmy *et al.,* 2015; Akashi *et al.,* 2005). Chickpea is also a good source of vitamin B3, hence used as vitamin supplement (Wood and Grusak, 2007). Methanolic extract of chickpea has aphrodisiac activity (Al-Snafi, 2016). Dose-dependent anti-inflammatory potential has been reported in extracts of chickpea (Shafeen, 2012).

1.11 CHEMICAL CONSTITUENTS OF CHICKPEA

Seeds of chickpea contain carbohydrate (59%), protein (29%), oil (5%), ash (4%) and fibers (3%) (Iqbal *et al.,* 2006). Chickpea is a best source of protein, and its one hectare produces 126 kg of protein, which is the third highest among legume crops after soya bean and ground nut. Starch is also the major component of chickpea seeds, which is organic long chain carbohydrate, and it helps to overcome many health aspects like diabetes, obesity, and digestion (Bray and Popkin, 2014). It is also a good source of amino acids, vitamins, iron, phosphorous, calcium, potassium, and magnesium (Akibode and Maredia, 2011). Moreover, chickpea is a main source of minerals such as calcium, magnesium, iron, zinc, potassium, selenium, and copper (Thavarajah, 2012). Minerals are major component of human diet and act as cofactors for different enzymatic activities (Ozlu *et al.,* 2021).

1.12 ECONOMICS IMPORTANCE OF CHICKPEA

Chickpea is grown and used for the following purposes:

1.12.1 Source of Food

Chickpea is highly nutritious legume food (Jukanti *et al.,* 2012). Chickpea is highly nutritive, and its seeds have high protein content (24–29%). Seeds of chickpea are consumed in fresh (green vegetables), as well as dried form (fried and roasted snack food). Its flour can also be used in making soup (Hulse *et al.,* 1989) and its paste is fried to make different dishes like "pakoras" (Younis *et al.,* 2019). Germinated seeds can also be eaten raw, as a salad ingredient. Young plants and green leaves are eaten in the same way that spinach is eaten.

1.12.2 Use as Fodder

In developing countries, chickpea is used as fodder for animals. Green or dried stems, leaves and gram husks are used as fodder for domestic animals (Younis *et al.,* 2019).

1.12.3 Use in Industries

In Turkey, chickpea starch is utilized to make a sticky material, which is used as adhesive in the plywood industries and used for the thickening of soups (Pradeep *et al.,* 1991). Starch of chickpea is used to brighten the wool and silk during final stage, in the

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textile industry. Its leaves are used to prepare a purple-bluish dye (Jambunathan *et al.,* 1989).

1.12.4 Role in Soil Health

Chickpea improves the health of soil by the fixation of atmospheric nitrogen into nitrates and nitrites. This nitrogen fixation is brought about in the nodules, present in the roots of chickpea and it is termed as biological nitrogen fixation (BNF). During this fixation, certain amount of nitrogen is accumulated in the soil (Khattak *et al.,* 2006). It is estimated that chickpea can fix 140 kg N ha^{-1}, in single growing season (Prajapati *et al.,* 2019). Furthermore, nitrogen is the major element for the metabolic activities of plant such as protein synthesis. The maximum nitrogen fixing ability of crop depends upon number of nodules, cultivar, and rhizobia, present in the root nodules.

1.13 CHICKPEA CULTIVATION IN PAKISTAN

The rain-fed Thal region (Punjab) is the major chickpea growing area of Pakistan. In Sindh and Baluchistan, chickpea is grown in residual moisture condition, after the harvest of rice crop. In Pakistan, among all pulses, 73% area (985 thousand hectares) is under the cultivation of chickpea, which give 673 thousand tones production (Economic survey of Pakistan, 2012-13).

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1.14 BIOTIC STRESSES IN CHICKPEA

Fungi are the most significant pathogens after viruses and bacteria that cause reduction in chickpea production. Almost 67 diseases in chickpea have been reported to be caused by 22 viruses, 3 bacteria and 80 nematodes, but only few of these diseases cause significant economic losses (Nene *et al.,* 1996). Fungal diseases (Ascochyta blight, Botrytis grey mold and Fusarium wilt) are famous for economic losses of chickpea. Blight and wilt are the most devastating diseases of temperate and tropical regions, respectively. Besides above mentioned diseases, most common chickpea diseases in the Mediterranean countries include stunted growth, stem and root rot (Nene *et al.,* 1989). The fungus *Ascochyta rabiei* causes Ascochyta blight. It is the most threatened diseases of chickpea and may causes 100% yield losses, in several cases. This disease is common in Greece,

Pakistan, Syria, Bulgaria and Tunisia. (Nene and Reddy, 1987). *A. rabiei* is a genetically unique that produces plant toxins such as solanopyrone A, B and cytochalasin D, that imparts role in the progression of disease (Wilson and Kaiser, 1995).

Fusarium oxysporum causes wilt disease in chickpea and results in significant yield losses. This highly destructive disease has been reported in Pakistan, Iran, Spain, India, Nepal, Mexico, Burma, Tunisia, Algeria, Syria, and Morocco (Kraft, 1994). *Fusarium oxysporum* f.sp. ciceri is a deuteromycetes fungal pathogen and it may cause 90% decline in crop production (Jimenez-Diaz *et al.,* 1989). *F. oxysporum* may cause severe damage in highly vulnerable cultivars, within 25 days of sowing in the field. The affected seedlings have drooped leaves and a pale appearance. Plant may fall and lie flat on the ground and die, eventually. The roots do not look to be decaying outside, however when dissected lengthwise as from lower nodes down, the internal tissues, particularly the pith and xylem, reveal a dark discoloration. The pathogen enters the roots of chickpeas and moves to the xylem, where it decreases or eliminates water delivery to the aerial portions (Halila *et al.,* 2009). Because the infection is soil-borne, crop rotation and chemical application are ineffective in combating it. Controlling the pathogen is particularly challenging due to its persistence in soil and capacity to thrive there for years, without a host (Haware *et al.,* 1996). It is problematic to control wilt disease because of the race diversity of *F. oxysporum.*

Botrytis cinerea is another dangerous and devastating chickpea fungus that causes grey mold. In Bangladesh, India, Pakistan, Nepal, Argentina, and Australia, crop damages of up to 100% have already been recorded under optimal circumstances. The disease has also been detected in a number of European states (Pande *et al.,* 2007). *B. cinerea* can attack approximately 100 species of plants of various genera, such as fruits, vegetables, ornamental plans and weeds. Black gram, carrot, cabbage, lettuce, eggplant, mung-bean, lentil, and other crops are the major hosts of this fungus. Under artificial inoculation conditions, it was found that *B. cinerea* isolated from chickpea may infect various other crops and weed species (Rathi and Tripathi, 1991). As a result, alternate hosts are expected to play a key impact in the pathogen's long-term field survival.

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Dry root rot is another important disease of chickpea. It is caused by a soil-borne fungus called *Rhizoctonia bataticola* (Taub). This disease has been found in Pakistan, India, Australia, Iran, Spain, Ethiopia, and US. The prominent symptoms of this disease appear in the chickpea fields, when flowers bloom and pods start to develop, the plant becomes dry and roots, leaves and stems becomes straw colored and can be broken easily. *M. phaseolina* can also cause root rot in a wide range of wild and cultivated species of chickpea, under a variety of environmental conditions.

Viruses also infect chickpea plant and cause a variety of diseases. Chickpea stunting and wilting are the most serious viral disease of chickpea. Kaiser and Danish (1971) were the first to report virus-induced wilting in chickpea, and Nene and Reddy followed suit in India (1987). Virus-induced wilting disease then spread to North Africa and West Asia, causing serious concern in Tunisia, Morocco and Lebanon. Formerly, almost around 80-100% yield losses have been reported by this disease (Horn *et al.,* 1996). Luteoviruses such as legume yellow virus, subterranean clover red leaf virus, and beet western yellows virus, have been reported to cause stunting in California (Bosque-Perez and Buddenhagen, 1990).

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1.15 ABIOTIC STRESSES OF CHICKPEA

Chickpea is exposed to a range of abiotic challenges during its life cycle, including dehydration, frost, extreme temperature, salinity, poor drainage, acidification, and metal toxicity (Millan *et al.,* 2006). Comparative study reveals that abiotic stress-related yield losses (6.4 million tonnes) may outnumber biotic stress-related yield losses (4.8 million tonnes). Drought/heat, cold, and salinity have caused substantial yield losses of 1.3 billion, 186 million, and 354 million dollars, correspondingly, in chickpea-growing countries (Ryan, 1997).

Chickpea is generally grown in dry and moderately tropical climates under monsoon conditions. Reduced rain and loss of soil water result in ultimate water deficit and may decrease up to 50% of its yield. Drought wreaks havoc on flower and seed production. Each year, terminal drought stress reduces chickpea production by up to 50%, over the world. Reactive oxygen species (ROS) generate oxidative stress by creating a hazardous environment for plants. The proper function of numerous metabolic pathways

in the cell is harmed by oxidative stress. Antioxidants like glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and ascorbate peroxidase (APX) enhance or decrease their activity in response to drought stress, depending on the plant's tolerance or susceptibility. Drought-tolerant plants store proline to counteract the effects of stress (Waqas *et al.,* 2019).

The production of the cool-season chickpea is low due to low temperatures. Temperatures in the freezing range of chickpeas are less than –1.5 °C, which is the freezing point of plant tissue. For winter-sown chickpeas, temperatures in the freezing zone are a major concern. In these places, freezing stress is most prevalent during the germination and early vegetative stages of crop growth. Isolated freezing episodes (frost) also affect chickpea crop during late vegetative growth and physiological maturity. Chilling temperatures, on the other hand, can reduce chickpea production and vigor at all phenological stages, but are likely to be most harmful to yield during reproductive phase (Croser *et al.,* 2003).

Chickpea grain development can be severely harmed by heat stress. Chickpea grain yield is regulated by temperature range and is associated to phenology. Chickpea productivity is hampered by high temperatures (less than 35 °C) during the reproductive phase. Temperatures above 30 °C lower grain weight and number. Chickpea yields are significantly reduced by even at 1 °C increase in temperature above the threshold. Significant yield loss in several chickpea cultivars have surpassed 100% at temperature rise. High temperatures have a substantial impact on chickpea podding, which may be attributed to a breakdown in source and sink relationships from green leaves to another tissue, which results in pollen grain death (Rani *et al.,* 2020).

In dry and semi-arid places, salinity has a detrimental effect on chickpea production (Ryan, 1997). Dua (1992) determined that chickpea viability under salt requires an electrical conductance (EC) threshold of 6 dS. Salt stress lowers water concentration (Munns, 2002), induces ion imbalance (Hassanein, 2000), and causes toxicity (Munns, 2002). In plants, salinity produces osmotic stress, ion toxicity, ion imbalance, nutrient insufficiency and poor nutritional status (Tejera *et al.,* 2006). Salt stress affects flavonoid

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coloration in the foliage of desi and kabuli chickpea cultivars (Millan *et al*., 2006). Saline conditions also influence flower and pod development, plant growth, photosynthesis, energy and lipid metabolism (Ramoliya *et al*., 2004; Katerji *et al*., 2001; Vadez *et al.,* 2012). Salinity has been reported to impact growth of plants, number of flower and pods, seed weight, and seed quantity in kabuli and desi varieties (Sohrabi *et al*. 2008). Flowers *et al*. (2010) reported deleterious impact of salinity on nodule formation, nodule size, and nitrogen fixation. Interestingly, both vegetative and reproductive stages are equally sensitive to salinity (Samineni *et al.,* 2011)

Both shoot ion independent and shoot ion dependent stresses limit growth and development of chickpea plants. Ionic stress arises several days or weeks after salinity stress, whereas shoot ion independent stress appears after ions gather in the shoot. Shoot ion independent stress is caused by the hydraulic barrier imposed by NaCl in the plant xylem, as well as a decrease in extracellular turgor pressure, which competes with water intake and slows plant growth. Salinity reduces growth rate, which eventually lead to a fall in shoot biomass. Salinity resistant chickpea cultivars can sustain high shoot biomass (Atieno *et al.,* 2017).

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1.16 SALINITY

Salinity is a term used in agriculture to describe the presence of salts more than what the plant requires (Yadav *et al.,* 2011). Salinity, often known as soil cancer, is a very deadly condition (Chandio *et al.,* 2017). This phenomenon has a negative impact on the production of a variety of crops. Salinity is usually identified by the creation of a white crust on the soil surface, like snow on the ground. The level of sodium contained in the crust is represented by the creation of white crust (Sonon, 2015). Various forms of salt-affected soils are used to alter nearly 10% of the total land surface. The extent and distribution of salt-affected soils has not been investigated in depth globally, until now. According to current estimates, the world's salinity-affected land area is over 1125 million hectares, with roughly 76 million hectares influenced by human-induced soil salinity and sodification (Wicke *et al.,* 2011). Salinity, on the other hand, affects one-fifth of agricultural fields. Meanwhile, 1.5 million hectares of very saline soils are becoming

unsuitable for agriculture. If soil salinization continues at this rate, half of the world's cultivable land will be destroyed by 2050. Australia, Bangladesh, China, Egypt, India, Iran, Iraq, Mexico, Pakistan, the former USSR, Syria, Turkey, and the United States are among the countries with critical salt-influenced soils (Hasanuzzaman *et al.,* 2014a).

1.17 SALINITY TYPES AND CAUSES

Salinity is usually categorized in following two major types (Parihar *et al.,* 2015):

1.17.1 Natural or Primary Salinity

Primary salinity is caused by natural processes such as the deposition of dissolved salt in the soil or underground water. Two natural phenomenon causes salinity, one is weathering that decays stones, producing dissolved salts such as sodium, calcium, and magnesium chlorides, and, to a lesser extent, sulfates, and carbonates. Sodium chloride is common among all. The other phenomenon is wind and rain-induced sea salt deposition. Ocean salts, chiefly NaCl, are transported inland by airflow and deposited by rainwater as "cyclic salts."

1.17.2 Human Induced or Secondary Salinity

Human activities disrupt the soil's physicochemical equilibrium between applied water (irrigation or rainfall) and crop water, resulting in secondary salinization (Garg and Manchanda, 2008). Land removal and the replacement of consistent foliage with crop production and irrigation systems, that employ salt-rich ground water or have inadequate drainage, are the most common causes.

1.18 IMPACTS OF SALINITY IN PLANTS

Salt stress affects agricultural crops in a number of different ways. Salinity not only affects the agriculture productivity of most of crops, but also deteriorates the physicochemical features of the soil and the area's natural ecosystems. Reduced agricultural production, lower economic benefits and soil damage are just a few of the negative effects of salinity (Hu and Schmidhalter, 2004). Germination percentage, seedling growth, and water and nutrient intake are all affected by salinity, which is caused by a complex combination of morphophysiological, and biological characteristics

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(Akbarimoghaddam *et al.,* 2011). Salinity affects plant development in almost every manner, particularly sprouting, seedling growth, and reproduction. As a result of soil salinity, water uptake is hindered and plants become sensitive to oxidative damage, osmolality, and nutritional deficiencies. Plant phosphorus (P) uptake is substantially reduced in salinity because phosphorus precipitates with calcium ions (Bano and Fatima, 2009). Plants are particularly sensitive to sodium, chlorine, and boron, for example. Excess sodium in cell walls can quickly raise osmotic pressure, leading to cellular damage (Munns, 2003). Even at low salt concentrations, crops become more vulnerable to toxic metal. High salinity levels in the soil might upset the plant's nutritional balance or prevent some nutrients from being taken because so many salts are easily available to plants (Blaylock *et al.,* 1994). Photosynthesis is also influenced by salinity, which reduces leaf area, chlorophyll content, transpiration rate and effectiveness of photosystem II (Netondo *et al.,* 2004). Salinity results in osmotic stress, so it imparts variety of negative consequences on plant growth and reproductive development (Ashraf, 2004). These conditions have detrimental physiological and biochemical effects on plant vegetative growth, as well as molecular implications (Munns and James, 2003; Tester and Davenport, 2003).

For plants developing in a saline media, osmotic stability is critical. Cells lose their turgidity, get dehydrated, and eventually perish when this balance is broken. On the other hand, the negative effects of salinity on plant growth could be attributed to the reduction in the transportation of minerals or hormones to the growing tissues (Ashraf, 2004). The substitution of Na^+ for K^+ in metabolic activities, as well as protein rearrangements mediated by Na^+ and Cl^-, generate ion toxicity. Na^+ cannot substitute K^+, which is a cofactor for many enzymes. Furthermore, elevated K^+ levels are necessary for tRNA to bind to ribosomes and consequent protein synthesis (Zhu, 2002). Osmotic damage is exacerbated by oxidative and osmotic stress, which causes metabolic imbalance (Chinnusamy *et al.,* 2006). Salt has a higher deleterious impact on plant development throughout the reproductive phase. Wheat plants exposed to NaCl generate fewer spikelet per spike, delay spike emergence, have lower fertility and give reduced grain yield. The amounts of Na^+ and Cl^- in the apical meristems of these seedlings, however, have been reported to be less than 50 and 30 mM, respectively, which were insufficient to impede

metabolic reactions (Munns and Rawson, 1999). As a result, salinity influences cell growth and division, which may be to blame for salinity's harmful impacts. Salinity briefly stops the cell cycle by down-regulating cyclin genes and cyclin-dependent kinases, ultimately reducing the growth. During salt stress, post-translational inhibition reduces the activity of cyclin-dependent kinase. Salinity also has an unfavorable effect on plant growth and development, preventing germination of seeds, enzymatic activities (Seckin *et al.,* 2009), synthesis of protein, RNA and DNA, and mitosis (2010; Javid *et al.,* 2011).

1.19 METHODS TO TACKLE SALINITY STRESS

Contemplating the detrimental influences of salt stress on plants, researchers are aiming to define approaches for tolerating salt stress in various crops. Many researchers have found that the use of exogenous phyto-protectants has a positive effect on alleviating salt-induced wheat damage. Some of the relevant information has been presented in this section.

1.19.1 Osmo-protectants

At various levels of organizations, plants show several adaptations to avoid the adverse consequences of various environmental stresses including salinity. Under the influence of salinity, plants synthesize and accumulate organic compatible solutes such as sugar to cope ionic, osmotic, and oxidative stresses. One of the most important physiological strategies utilized by plants during salt stress is the accumulation of these suitable solutes. Osmolytes are small, highly soluble, non-toxic chemical molecules that assist organisms in dealing with osmotic stress (Ashraf and Foolad, 2007). Osmo-protectants include:

i. Alpha-amino acids, for instance, ectoine and proline.
ii. Ammonium compounds like beta-alanine betaine, glycine betaine (GB), dimethyl sulfoniopropionate (DMSP) and choline.
iii. Sorbitol, sugars, trehalose, polyols, sugars and mannitol. Both osmotic adjustment and membrane stabilization rely on these osmo-protectants. As a result, plants that over-express osmo-protectant production and metabolic genes have better salt stress resistance (Hasanuzzaman *et al.,* 2017).

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1.19.2 Plant Hormones

Plant hormones are chemicals released at low concentrations within plants that stimulate plant growth and resistance under the influence of various abiotic stresses, such as salt stress. Various forms of plant hormones, such as abscisic acid, indole-3-acetic acid, cytokinin, gibberellic acid and brassino steroids are now utilized externally to relieve different environmental stresses, including salt stress (Ryu and Cho, 2015).

1.19.3 Plant Nutrients

Plant nutrients play a beneficial role, along with other physiological and biochemical characters, in relieving the adverse consequences of environmental stresses. The exogenous application of potassium (K) improves the growth and development of wheat seedlings under the influence of salt stress by enhancing antioxidant enzymatic activities, chlorophyll a, b and carotenoid activity and by decreasing the uptake of sodium and potassium (El-Lethy *et al.,* 2013; Kausar and Gull, 2014). Foliar application of phosphorus (P) also ameliorates the harmful effects of salinity by increasing plant growth attributes and decreasing sodium uptake (Khan *et al.,* 2013). Under salinity circumstances, calcium sulfate boosts plant growth, water status, and calcium and potassium uptake (Zaman *et al.,* 2005). Calcium nitrate reduces malondialdehyde generation and relative electrolyte leakage in wheat seedlings, minimizing oxidative damage induced by salt (Tian *et al.,* 2015).

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1.19.4 Antioxidants

To decrease ROS level, antioxidants are essential for plants. In their cellular components, plants have numerous non-enzymatic antioxidants to defend themselves from oxidative stress. AsA, GSH, tocopherol and other phenolic compounds are among the main antioxidants. Some of these antioxidants demonstrate advanced safety when applied exogenously against salt-induced oxidative stress (Hasanuzzaman *et al.,* 2017).

1.19.5 Seed Priming

Seed priming is a type of seed treatment where the seeds are immersed and then desiccated to their initial weight before the germination process begins, ensuring that no dramatic protrusion occurs (Khan, 1992). In ornamental plants, vegetables and field crops,

seed priming has the dual benefit of promoting significant and consistent proliferation of plants and obtaining higher grain yields (Dearman *et al.,* 1987; Farooq *et al.,* 2008). (Hartz and Caprile, 1995). Seed priming has dual effect of enhancing consistent and significant seedling growth as well as achieving greater yield (Farooq *et al.,* 2008).

Seed pretreatments aid seed growth in stressful situations (Welbaum *et al.,* 1998). Species of plants, priming duration, temperature, priming agent's water concentration, seed vigor, dehydration, and the conditions in which primed seeds are stored are all factors that combine to determine seed priming success (Parera and Cantliffe, 1994). Maize, wheat, chickpea, sorghum, rice, and other crops have been tested for priming (Harris *et al.,* 1999). Success of seed priming depends on plant species, priming methodology, chemical concentration, plant growth stage, and maturity period (Tzortzakis, 2009).

1.19.5.1 Beneficial Aspects of Seed Treatment

Following are some advantages of seed treatment:

i. Elevated antioxidant enzymes activity is observed in primed seeds (Wang *et al.,* 2003, McDonald, 1999, Hsu *et al.,* 2003).
ii. Seed priming is involved in membrane repairing, improves the process of protein synthesis, and helps to repair and strengthen nucleic acids.
iii. After priming, glyoxysome activity is increased (Lin and Sung, 2001).
iv. An early flower emergence and resistance to the cool atmosphere can be achieved by various priming methods in areas where the temperature is low and the farming time is limited (Hoseini *et al.,* 2013).
v. In those areas where farming is delayed due to the shortness of time, seed priming is suggested.
vi. During winter, seed priming decreases the consumption of water due to premature plant development.
vii. Seed priming increases tolerance in plants by showing best results in unfavorable conditions.
viii. Priming improves the process of seedling development and the process of germination.

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ix. It also improves the process of root development.
x. In lettuce, seed priming breaks seeds dormancy (Chastokolenko, 1984).

1.19.5.2 Types of Priming

There are several different types of seed priming techniques including:

i. Hydro-priming: It involves dipping seeds in water.
ii. Osmo-priming: It entails immersing seeds in organic solvents for a period.
iii. Halo-priming: Seeds are immersed in inorganic dissolved salts.
iv. Thermo priming: Seed are kept at low and high temperature before sowing.
v. Bio priming: Use of biological agents for seed priming.
vi. Solid matrix priming: Solid matrix seed treatment (Ashraf and Foolad, 2005).

1.19.5.3 Bio-priming

It is one of the new technique of seed treatment which helps seeds to germinate even under adverse stress conditions. Bio-priming is about treating seeds with a biological substance and then re-drying them, which starts the germination process except for radicle emergence. Bio-priming is a method of hydration that involves the use of any biological ingredient (Ashraf and Foolad, 2005). Bio-priming has been utilized to improve the germination rate and consistency (Reddy, 2012). Seeds can be bio-primed with a beneficial microbial suspension for a long time, allowing the absorption of microbial agent in seed (Abuamsha *et al.,* 2011). It involves the application of advantageous microorganisms. It increases speed and consistency of germination and quality and yield of crop. Reddy (2012) clarified that hydration of seeds followed by the use of beneficial bacterial suspension to the seeds (bio-priming) secure seeds against infection This bacterial inoculum initiates the physiological procedures where plumule and radicle development of seed is avoided, until the point when seeds have sufficient oxygen and favorable temperature. Plant growth promoting rhizobacteria (PGPR) continue duplicating in the seed and multiply even before sowing (Taylor and Harman, 1990). Bio-priming treatment advances germination of seed and promotes improved plant development under various stresses (Moeinzadeh *et al.,* 2010).

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Bio-priming is considered as a simple, environment friendly, long-lasting and efficient seed treatment (Mahmood *et al.,* 2016). Though different researchers have enlightened the significance of bio-priming (Mirshekari *et al.,* 2012), it is still an unclear approach and needs to be explored and debated. Diverse strategies have been utilized that explain bio-priming and the difference in these strategies is in the optimum temperature and soaking interval (Kasim *et al.,* 2013).

1.20 PLANT GROWTH PROMOTING RHIZOBACTERIA

Microorganism populations lives in rhizosphere and display an intimate interaction with the root zone of plants. Usually, the bacterial community is larger here than in other regions of the soil. Thus, the plant root is considered to be a significant supplement for microbes, colonizing the rhizosphere. Plants release organic carbon into the environment through root exudates, which offer nutrition to rhizobacteria (Hardoim *et al.,* 2008). PGPR is a type of soil bacteria that colonize the plant roots in the rhizospheric area and promotes the growth of plant (Hayat *et al.,* 2010).

Although the precise mechanisms for stimulating plant growth remain largely speculative, potential theories include:

i. Hormone synthesis such as ABA, GA3, CK and IAA.

ii. The manufacture of important 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzymes to reduce ethylene levels in the root of developing plants.

iii. Fixation of nitrogen.

iv. Manufacture of siderophores.

v. Solubilization of nutrients, particularly mineral phosphate, and mineralization.

vi. Enhancement of tolerance to abiotic stresses (Hayat *et al.,* 2010).

1.21 MECHANISM OF ABIOTIC STRESS RESPONSE BY PGPR

Various abiotic stresses including heat stress, cold stress, dehydration and salinity result in metabolic virulency, membrane disorder, reactive oxygen species generation,

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photosynthesis inhibition, decreased nutrient accession and different levels of hormones. Accumulation of osmolytes, development of radical removing mechanisms for superoxide, ions elimination or compartmentalization by effective carrier and symporter approaches, and synthesis of unique enzymes required for plant hormone adjustments are some of the mechanisms produced by plants to respond to various environmental stresses (Parida and Das, 2005; Shao *et al.,* 2009).

1.21.1 Synthesis and Modulation of Plant Hormones

Plants are immobile species with high physiological adaptability, which enables them to survive a wide range of environmental stresses. This is due to the constant meristem activity of roots and shoots, and embryogenesis (Wolter and Jurgens, 2009). They have developed a variety of stress responses that involve root morphological modifications. Plant root structure is defined by root branching pattern and the speed and direction of individual root growth, and it is a suitable solution for comprehending how embryonic plasticity is translated into growth reactions (Malamy, 2005).

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1.21.1.1 Auxin Production

Indole-3-acetic acid (IAA) is a biologically highly effective auxin in plants. Importance of auxin in crop growth can be emphasized by the fact that no entirely auxin-deficient species exists on the earth. Auxin biosynthesis has a higher capacity not only in matured apical parts but also in roots, especially in the apical meristem (Teale *et al.,* 2006). Plant development, particularly the creation of root systems and branching is influenced by the production of Auxins (Potters *et al.,* 2009). Variations in auxin tolerance mediate changes in the organization of lateral root development and differentiation in *A. thaliana*. These changes affect auxin-responsive gene expression and increase pericycle cell proliferation (Pérez-Torres *et al.,* 2008).

High auxin accumulating mutants produce excessive lateral roots, whereas mutant plants with altered auxin distribution produce branched roots. Auxin may operate as a mediator between the impact of a level of stress and the manifestation of the response phenotype, as lateral root development is triggered by a wide range of abiotic stimuli.

Several mechanisms have been proposed to describe alterations in auxin metabolism and/or responsiveness induced by stress; however, evidence for alterations in signal transduction and catabolism induced by stress is mainly found in the literature. Water stressors, for example, influence transport of auxin by modifying PIN gene expression and/or limiting the transportation of lateral auxins (Potters *et al.,* 2009). The hydrolases that come from auxin conjugates have also been discovered to play a function in the plant's reaction to stress (Muller, 2011). In Arabidopsis, upregulation of auxin-amidohydrolase is linked to greater salt stress tolerance and lower root elongation inhibition. This effect could be attributed to a significant increase in free auxin content to defend against saline conditions (Junghans *et al.,* 2006).

More PGPR strains that generate IAA were identified when plant tissues were tested for bacteria (Spaepen *et al.,* 2008). Plants injected with these microorganisms show improved lateral root and root hair formation, as well as increased root growth (Bharti *et al.,* 2020). The *Azospirillum* strains enhance root growth by the production of phytohormones (Spaepen *et al.,* 2008). Inoculation of *A. brasiliense* in soybean and rice seedlings increases the growth attributes of roots when compared with uninoculated control (Molla *et al.,* 2001). The response of bean roots treated with IAA was similar to that of roots inoculated with *Azospirillum* (Remans *et al.,* 2008).

1.21.1.2 Ethylene Production

It has been shown that plants generate ethylene in response to stressful stimuli at two different levels. At first level, lower concentration of ethylene upregulates stress tolerant genes, while at second level, higher concentration of ethylene is produced that restricts the growth of plants and initiates yellowing, aging process in plants and dropping of different parts of plants. Growth inhibition and adverse effects on plants, such as senescence, chlorosis, and abscission are aided by the greater quantity of ethylene secreted in phase 2 (1-3 days after stimulation) (Glick *et al.,* 2007). The ethylene precursor (ACC) is converted by bacterial ACC-deaminase into 2-oxobutanoate and ammonia, lowering ethylene levels in plant roots, reducing ethylene repression of auxin reaction component synthesis, and thereby promoting plant growth (Kang *et al.,* 2010).

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1.21.1.3 ACC Deaminase Production

It was suggested that roots of plant release ACC and convert it into ACC deaminase by bacteria, present in the soil. As a result, bacterial growth will be stimulated by hydrolyzed ACC products. The ACC-deaminase feature between plants and PGPR seems to be mutually beneficial as continuous ACC secretion and bacterial degradation can reduce ethylene in plants (Glick *et al.,* 1998).

Plant growth promoting bacterial strains expressing ACC deaminase are useful in defending a number of plant species against a variety of stressors. According to Mayak *et al*. (2004), *Achromobacter piechaudii* with ACC deaminase activity considerably boosted the fresh and dry weight of tomato seedlings cultivated in the presence of NaCl salt (up to 172 mM). In contrast to plants inoculated with Pseudomonas strains without ACC deaminase activity, *Pseudomonas fluorescens* strain TDK1 expressing ACC deaminase activity displayed salt tolerance and improved yield in groundnut plants (Saravanakumar and Samiyappan, 2007). Pseudomonas generating ACC deaminase and indole acetic acid protected canola plant development under saline circumstances by lowering ethylene levels (Siddikee *et al.,* 2010). Under saline stress circumstances, growth properties were reported to be boosted in maize and pea plants after inoculation with ACC deaminase producing *Pseudomonas fluorescens* and *Pseudomonas spp* (Kausar and Shahzad, 2006; Ashad *et al.,* 2008). Salt stress in wheat has been reported to be mitigated by ACC deaminase secreting rhizobacteria (Nadeem *et al.,* 2010).

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1.21.2 Accumulation of Nitrogen Containing Compounds (NCC)

The NCC start accumulating in plants under salinity stress, thereby initiating the stress tolerance mechanism (Parida and Das, 2005). Amides, amino acids, proteins, polyamines and fumaric acid are the most widely accumulated NCCs. Many plant species have been found to accumulate a lot of proline in their cells when they are stressed by salt or drought (Szabados and Savourè, 2009). Proline synthesis has a multifaceted effect on growth of transgenic plants and stress responses. Proline has been identified as an osmolyte capable of storing carbon and nitrogen. Saline and drought stress are known to disrupt the balance of free radicals and antioxidants. Proline acts as a molecular chaperone, helping to

stabilize protein structures and improve enzyme activity. Proline aggregation aids in the maintenance of cytosolic pH and the regulation of redox potential within the cell (Verbruggen and Hermans, 2008).

Enhanced proline production has been observed in plants treated with different plant growth promoting rhizobacteria under the influence of various abiotic stresses (Vardharajula *et al.,* 2011). Energy expenses (41 moles of ATP) are required for the creation of proline and other suitable solutes, and they happen at the expense of crop production. They may also enable plants to survive and thrive under excessive salt stress (Munns and Tester, 2008).

1.21.3 Antioxidant Enzymes Activity

Antioxidant enzymes are the by-products of various metabolic activities of various compartments of the cell (Apel and Hirt, 2004). The ability of these species to generate cell damage to DNA, proteins, and lipids is a common trait. As the internal oxygen concentrations in chloroplasts are high during photosynthesis, they are more likely to trigger higher production of oxygen species (Gill and Tuteja, 2010). Several anti-oxidative protection products scavenge these molecules, which are normally limited to specific components under physiological steady-state conditions (Apel and Hirt, 2004). Under normal growth conditions, the synthesis rate of cellular ROS is low, but it increases during stress. The difference between the generation and scavenging of ROS causes ROS buildup during stress. SOD, APX, and CAT enzymes are important ROS-scavenging molecules in plants. Antioxidants present in high concentrations in chloroplasts and other cellular compartments, such as ascorbic acid and glutathione, are also essential for plant defense against oxidative stress (Das and Roychoudhury, 2014). The overall balance of antioxidants is critical for determining the stable proportion of superoxide radicals and hydrogen peroxide for the removal of excess ROS in plants and must be properly regulated (Mittler, 2002).

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In lettuce plants treated with PGPR strains, the synthesis of peroxidase and catalase is implicated in the relief of salt stress (Kohler *et al.,* 2010). Under non-saline conditions, treatment with *P. mendocina* resulted in equivalent increase in plant growth. Salinity

lowered dry mass of lettuce roots and shoots. Both at low and mid salt concentrations, the plants inoculated with *P. mendocina* had considerably greater shoot weight than the control plants (Bianco and Defez, 2009). The ability of PGPRs to mitigate the effects of drought stress on maize has also been documented. Drought stress was encountered by various species of Pseudomonas including *P. putida, P. entomophila, P. syringae, P. stutzeri,* and *P. montelli.* These strains improved the growth and activities of osmolytes and ROS scavenging enzymes (Sandhya *et al*., 2010). A decrease in antioxidant enzyme activity has also been reported in barley plants, under salinity stress. Salinity caused a considerable increase in POD and CAT activity in salt-stressed leaves of two different salt resistant cultivars of barley (Omar *et al*., 2009).

1.21.4 Increase of Nutrients Uptake

Crop's ability to adapt defensive measures to alleviate or survive stress is critical to their long-term viability and efficiency (Munns and Tester, 2008). Plant nutrient buildup is linked to how they respond to diverse environmental conditions. The negative effects of abiotic stresses are exacerbated by plant mineral nutrition deficiency, and high quantities of macronutrients are intentionally provided to lessen the detrimental effect of stress on growing plants (Khoshgoftarmanesh *et al.,* 2010).

Phosphorous is required for plant production after nitrogen. It is an essential component of various nucleotides, phosphoproteins, nucleic acids and phospholipids. Mostly, salinity results in decrease of phosphorus content (Parida and Das, 2005). Saline soil has less availability of phosphorus because of ionic strength, which decreased phosphate activity and concentrations of phosphorus through absorption and low calcium-phosphorus mineral solubility. In saline soil, soluble phosphorus concentrations are typically 1 ppm or less (Hisinger, 2001). Hydrogen phosphate and dihydrogen phosphate are the accessible forms of phosphorus. Phosphorus occurs as organic and inorganic phosphate in two types in the soil, and it has restricted mobility in the soil like other nutrient elements such as K, Fe, Zn and Cu (Hayat *et al.,* 2010). A fundamental feature of PGPR strains is the transformation of insoluble phosphate molecules into a plant-accessible form. Different kinds of plant growth-promoting bacteria exhibit phosphate-solubilizing action

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(Khan *et al.,* 2009). The fundamental mechanism of mineral phosphate solubilization is the action of organic acids generated by soil microbes. The synthesis of organic acids causes the microbial cell and its surroundings to become acidic. Consequently, P can be released by proton replacement for Ca^{2+} from a mineral phosphate. Various plant growth promoting rhizobacteria belonging to various genera, like *Bacillus, Pseudomonas, Rhizobium* and *Erwinia* can synthesize various organic acids (Carmen and Roberto, 2011).

1.22 AGRICULTURAL IMPORTANCE OF SALT STRESS TOLERANT GENES IN MITIGATION OF SALT STRESS

Salt is currently affecting more than 800 million hectares of land, around the world, which could dramatically reduce crop production (Munns and Tester, 2008). Salt stress relief mechanisms include the production of salt tolerant varieties, seeping of excessive dissolved salts from the higher to lower depths of the soil, rinsing of soils containing soil surface crusts, elimination of salt in regions with significant water supply or rainfall, and by cultivating salt-accumulating plants (Bacilio *et al.,* 2004). By inoculating crop seeds and seedlings with beneficial bacteria is an alternate approach to decrease salt stress. Hydraulic conductance, accumulation of osmo-protectants, toxic Na+ ions sequestering, higher stomatal conductance maintenance and photosynthesis have been correlated with the beneficial effect of PGPB under salinity (Dodd and PerezAlfocea, 2012). Various studies revealed the enhancement of salt tolerance by transforming salt tolerant bacterial genes in crops of agricultural significance (Table 1.1). Bacteria of agriculture importance have been listed in Table 1.2.

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Maintaining cellular ion homeostasis by reducing damaging sodium (Na^+) concentrations is one of the most critical responses to salt stress (Tester and Davenport, 2003). The salt overly sensitive (SOS) signaling pathway is a well-established signaling mechanism that is known to have a function in ion homeostasis maintenance. At the molecular level, the SOS signaling pathway has been identified as an important mechanism for Na+ elimination and ion homeostasis regulation (Zhu, 2000). SOS proteins include SOS1, SOS2, and SOS3. SOS1 regulates Na^+ outflow at the cellular level by encrypting the Na^+/H^+ antiporter in the plasma membrane. The sodium ion transfer between the

vegetative parts of plant is also controlled by SOS1. When this protein is over-expressed, salt tolerance is improved (Shi and Zhu, 2002). The Ca^{2+} signals that are activated by salinity activate SOS2. The SOS2 gene provides instructions for making serine kinase/threonine kinase, which controls domain C-terminal and the catalytic domain N-terminal (Liu *et al.,* 2000). SOS3 is a protein that binds to myristoylated Ca^{2+} and has a site on its N-terminal for myristoylation. In fact, this site plays a prominent part in providing tolerance against salt stress (Ishitani *et al.,* 2000). In SOS2 protein, C terminal's regulatory domain has NAF domain, also defined as the FISL motif. This domain is made up of roughly 21 amino acids and serves as a binding site for Ca^{2+-} binding SOS3 protein. Protein kinase promotes the interaction of SOS2 and SOS3, which then phosphorylates SOS1 and improves its transport activity (Guo *et al.,* 2004). These proteins control the passage of membrane vesicles, pH, homeostasis, and vacuole activities in addition to salt tolerance (Quintero *et al.,* 2011). An increase in Na^{+} concentration is responsible for the rise in Ca^{2+} concentration, which connects the protein to SOS3. Ca^{2+} as well as intracellular Na^{+} homeostasis influence SOS proteins. As the SOS3 protein loses its self-inhibition, it associates with and activates SOS2 proteins. The SOS3-SOS2 complex is then transported into the plasma membrane, where SOS1 is phosphorylated, resulting in an increase in Na^{+} inflow and hence a reduction in sodium ions toxicity (Martinez-Atienza *et al.,* 2007). An effective technique is present in so many crops that regulate the ionic concentrations in their cells to that very low level. During the stress cycle, the membranes as well as its associated parts are responsible for maintaining the ionic concentration of cytosol by controlling ion intake and transport. Symporters, antiporters and ion channels are examples of carrier proteins that help with ion transport. Sustaining sodium and potassium ions balance in saline environments is critical for plant viability (Sairam and Tyagi, 2004).

The WRKY gene family is a plant-specific transcription factor (TF) that is involved in a variety of abiotic stress response pathways (Li *et al.,* 2020). The gene AtNHX1 expresses a vacuolar antiporter Na^{+}/H^{+} in *A. thaliana*, which is critical for salinity tolerance (Shi and Zhu, 2002). mHAK4 and ZmHKT1 (a HKT1 family Na^{+}-selective transporter) confer distinct roles in promoting shoot Na^{+} exclusion and salt tolerance (Zhang *et al.,* 2019).

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Table 1.1: Various salt stress tolerant genes from agriculturally essential bacteria.

S. No.	Gene	Bacteria	References
1.	*Kat*E, HPT and NPTII	*Escherichia coli*	Prodhan *et al.,* 2008
2.	EctABC	*Bacillus halodurans*	Kuhlmann and Bremer, 2002
3.	proH, proJ and proA	*Halobacillus halophilus*	Saum and Muller, 2007
4.	AcdS	*Hallobacillus* sp. SL3 and *Bacillus halodenitrificans* PU62	Ramadoss *et al.,* 2013
5.	Coda	*Arthrobacter globiformis*	Goel *et al.,* 2011
6.	BetS	*Sinorhizobium meliloti*	Boscari *et al.,* 2002
7.	OpuC and OpuB	*Listeria monocytogenes*	Fraser *et al.,* 2000

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Table 1.2: Some agriculturally important bacteria and their role in different plants

S. No.	Bacteria	Role	References
1.	Species of *Hallobacillus Bacillus* isolated from saline soil	Improve the growth of wheat seedlings through direct or indirect mechanisms under saline stress.	Ramadoss *et al.,* 2013.
2.	*Brevibacterium epidermidis* RS15, *Micrococcus yunnanensis* RS222, and *Bacillus aryabhattai* RS341 isolated from coastal soil	Noteworthy increment in growth attributes of canola plant.	Siddikee *et al.,* 2010.
3.	*Escherichia coli*	Mitigate salt stress in Indian rice.	Prodhan *et al.,* 2008.

S. No.	Bacteria	Role	References
4.	*Zhihengliuela alba, Bacillus licheniformis* and *Brevibacterium iodinum,* and isolated from coastal soil	Improvement of growth and salinity resistance in red pepper seedlings.	Siddikee *et al.,* 2011.
6.	Various species of *Bacillus* isolated from salt exposed rhizospheric soil	Nitrogen fixation and increased salinity resistance in wheat.	Upadhyay *et al.,* 2009.
7.	*Arthrobacter globiformis*	Elevated resistance to salinity and drought in tomato seedlings.	Goel *et al.,* 2011.

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All eukaryotes contain small guanosine triphosphate (GTP)-binding proteins with molecular weights ranging from 20 to 30 kDa that play a variety of activities (Lowy and Willumsen, 1993; Bourne *et al.,* 1990). Rab1, Rab2, Rab5, Rab6, Rab7, Rab8, Rab11, and Rab18 are the eight plant Rab subfamilies. They contribute to hormonal trafficking during fruit ripening and apical dominance, as well as brassinosteroid production, pollen and nodular growth, and stress response (Agarwal *et al.,* 2009). Rabs are the most diverse group of tiny GTP-binding proteins. Various environmental factors have different effects on the Rab genes. In *M. crystallinum*, a small GTP-binding protein of the Rab5 family was stimulated by 400 mM NaCl (Bolte *et al.,* 2000). Chill, dryness, and salinity stress treatments cause OsRab7 cDNA transcript to accumulate modestly. Under extreme biotic and abiotic stress, the Arabidopsis Rab7 gene was activated (Shanmugam *et al.,* 2013). Rab7 was also upregulated in exposure to environmental stresses including salinity in *Pennisetum glaucum* (Agarwal *et al.,* 2008). Rab7 transcript upregulation in response to various environmental stimuli implies that this protein is involved in stress adaption. Transgenic Arabidopsis with continuous overexpression of AtRab7 demonstrates enhanced resistance to saline conditions, as well as lower buildup of ROS under salinity stress (Mazel *et al.,* 2004). Tobacco transgenics overexpressing PgRab7, on the other hand, demonstrated improved resistance to drought and salt stress (Agarwal *et al.,* 2008).

1.21 AIMS AND OBJECTIVES

The main objectives of this study are as follows:

- Collection of soil samples from Khewra salt mine, located in Jhelum, Pakistan and analysis of their physiochemical properties.
- Isolation of halotolerant bacterial strains from rhizospheric soil samples.
- Identification and characterization of isolated bacteria and their application for salinity tolerance.
- ACC-deaminase, IAA and EPS activities analyses of bacteria in plant growth promotion and stress alleviation of Wheat and Chickpea.
- Identification of genes implicated in the tolerance to salt stress.

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2. MATERIALS AND METHODS

2.1 EXPERIMENT 1: ISOLATION OF HALOTOLERANT BACTERIA FROM RHIZOSPHERE OF KHEWRA SALT MINE HALOPHYTES AND THEIR APPLICATION TO INDUCE SALT TOLERANCE IN WHEAT

2.1.1 Collection and Processing of Soil Samples

Soil samples from rhizosphere of *Justicia adhatoda* (IS1)*, Chenopodium murale* (IS2) and *Cenchrus ciliaris* (IS3) were collected from Khewra salt mine, Pakistan (32° 37' 44.1" N and 73° 00' 47.1" E). Samples were taken from 0~20 cm depth in clean labelled polythene bags. All soil samples were positioned in an ice container and carefully brought to Molecular Plant Pathology Laboratory, Department of Plant Sciences, Quaid-i-Azam, University, Islamabad. Soil samples were crushed using mortar and pestle, shade dried and sieved to remove large stones, gravel, and other wastes.

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2.1.2 Physiochemical Properties of Soil Samples

Following properties of collected soil samples were examined:

2.1.2.1 Soil Organic Matter

A standard protocol was followed for the measurement of soil organic matter (Nelson and Sommers, 1983). Air dried soil was weighed (2 g) was transferred to the flask. Carefully, concentrated H_2SO_4 (20 ml) was added to the flask. In this mixture 1 N $K_2Cr_2O_7$ (10 ml), water and ortho-phospohoric acid (10 ml) were also transferred. After 30 minutes, few drops of diphenylamine, as an indicator were also added. The titration of mixture was performed against 0.5 N Mohr’s control.

2.1.2.2 pH

For the determination of soil pH, the protocol of McLean (1983) was followed. For this purpose, soil was mixed in water (1:1 ratio) and a pH meter (Russell RL060P) was used to measure the pH of suspension.

2.1.2.3 Electrical Conductivity (EC)

The protocol of McLean (1983) was followed to measure the electrical conductivity of each soil sample. For this purpose, soil of each sample was mixed separately in deionized water in 1:9 ratios. Stirring of suspension was performed for 8-10 minutes, until uniform turbidity. EC of these soil mixtures was measured by using electrical conductivity meter (DOS-11 AW) and described in milli siemens per meter.

2.1.3 Nutrients Analysis

For nutrient analysis, the methodology of Estefan *et al*. (2013) was followed. The atomic absorption spectrophotometer (AAS) was used to measure the concentration of different macronutrients such as Potassium (K), Nitrogen (N) and Phosphorus (P).

2.1.4 Isolation of Salt Tolerant Bacterial Strains

To make a soil suspension, each soil sample (1 g) was diluted in 25 ml of double distilled water, which was further diluted by 10-1000 folds and plated on Luria-Bertani (LB) media, supplemented with 2% NaCl. After the appearance of various colonies, each distinct colony was sub-cultured for further experimentation.

2.1.5 Salt Tolerance Ability of Isolated Bacterial Strains

The tolerance potential of isolated bacteria was checked at various concentrations of sodium chloride (NaCl). The bacterial strains were separately cultured on LB medium amended with various concentration of NaCl, ranging from 2-10%.

2.1.6 Morphological Identification

Pure colonies of salt tolerant bacterial strains were cultured on LB media and incubated for 24 hours. Colonies were observed for their shape, color and texture. The strains were gram stained by following the protocol of Vincent (1970) and examined under light microscope. The shape of the bacterial cell and motility was recorded.

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2.1.7 Molecular Characterization

For the molecular characterization of the selected bacterial strains, their DNA was isolated by CTAB method. After DNA extraction, under standard amplifying conditions, the 16S rRNA gene was successfully amplified using polymerase chain reaction (PCR). The Bio-Rad T100 PCR thermal cycler was used to amplify the selected gene using universal forward (27 F) and reverse (1492 R) primers. To the PCR master-mix, 20-30 ng of genomic DNA and 10 mM of each forward and reverse primer were added. The cycling conditions were 94 °C for 4 minutes, followed by 34 cycles of 94 °C for 40 seconds, 55 °C for 50 seconds, 72 °C for 45 seconds, and a final extension step of 72 °C for 4 minutes. Amplified PCR products were sequenced and used for BLAST analysis (http://www.ncbi.nlm.nih.gov) to determine sequence similarities. Six sequences were obtained and submitted into the GenBank NCBI database and accession numbers were obtained.

2.1.8 Phylogenetic Analysis Using Mega 7.0

For the phylogenetic analysis, MEGA version 11 was used. The Neighbor-Joining method was used to build the evolutionary tree of all sequences. The evolutionary distances were compared using Maximum Composite Likelihood techniques.

2.1.9 Antibiotic Resistance

Disk diffusion technique was employed to check the antibiotic resistance of selected bacterial strains (Kotková *et al.,* 2019). For this purpose, 100 µl of 24-hour old bacterial cultures were swabbed onto the solidified LB medium. Paper discs were dipped in antibiotics (viz. Neomycin, Penicillin, Streptomycin, Kanamycin, Rifampicin, Ampicillin, Chloramphenicol and Erythromycin) and placed on the swabbed media. Plates were sealed and positioned in an incubator at 35–37 °C for 24 hours. Area of the inhibition was measured to see the resistance of bacteria to specific antibiotic, and it was termed as "the zone of inhibition". Acquired data were arranged using the Kirby Bauer chart. Selected bacterial strains were classified as sensitive (S), intermediate (I), and resistant (R), in response to various antibiotics.

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2.1.10 Plant Growth Promoting Activity Assay

Standard protocols were followed for biochemical characterization and activity analyses of different enzymes including catalase (Denizci *et al.,* 2004), oxidase (Gerhardt *et al.,* 1981), protease (Ashwini *et al.,* 2011), amylase (Namasivayam *et al.,* 2011), cellulase (Naseem and Bano, 2014), pectinase and chitinase (Chenniappan *et al.,* 2019).

2.1.10.1 Catalase Activity

New colonies of bacterial isolates were deposited (one at a time) on microscope slides using sterile loops to measure the catalase activity. Then, a few drops of 2% H_2O_2 were added. The formation of bubbles, because of the oxygen generation, was evidence of active catalase activity.

2.1.10.2 Oxidase Activity

The Gerhardt *et al*. (1981) filter paper spot technique was used to measure the oxidase activity. A tiny strip of filter paper was soaked in rhizobacterial culture for 24 hours. On the culture, one or two drops of Kovacs oxidase (1%) reagent were applied. A change in color from light purple to dark purple over the course of 60 to 90 seconds allowed us to identify the presence of oxidase activity.

2.1.10.3 Chitinase Activity

Bacterial isolates were screened on the colloidal chitin agar medium, which was made by combining Na_2HPO_4, KH_2PO_4, NH_4Cl, NaCl, yeast extract, and 1 % (w/v) colloidal chitin with 15 grams of agar and 6 grams of Na_2HPO_4. The plates were incubated at 37 °C for 96 hours and areas of bacterial hydrolysis zones were recorded (Ramirez *et al.,* 2004; Lopes *et al.,* 2008).

2.1.10.4 Protease Activity

On skim milk media, the activity of proteolytic bacteria was tested, qualitatively. Skim media was prepared by dissolving skim milk powder (28.0 g), yeast extract (2.5 g), tryptone (5.0 g), agar (15.0 g) and dextrose (1.0 g) in 1000 ml of distilled water. Indications that the bacterial strains may incorporate protein (casein) were displayed by clear zones around the colonies.

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2.1.10.5 Cellulase Activity

Cellulase activity of selected bacteria was examined by spot inoculating bacterial strains onto LB agar, enriched with cellulose (10 g) and incubating them for 8 days at 28 °C. The rhizobacteria colonies that were encircled by halo zones were considered to have cellulase activity.

2.1.10.6 Pectinase Activity

5 g of pectin, 6 g of disodium hydrogen phosphate, 2 g of ammonium sulfate, 1 g of yeast extract, 3 g of potassium dihydrogen phosphate, and 20 g of agar were dissolved in 1 L of distilled water to prepare a media for the analysis of this activity. The solution's pH was raised to 6.0. The ready media was added into petri plates after the solution had been autoclaved. On the solidified media, bacterial colonies were spot-inoculated, and the petri plates were then incubated at 37 °C for 24 hours. Colonies of grown bacteria were stained with 50 mM iodine. Pectin dehydration was visible in the clean zone that formed around the bacterial colonies.

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2.1.10.7 IAA Production

Bacterial strains were cultured for 24 hours on 0.1% tryptophan treated LB broth media to measure auxin production. The growing media was centrifuged at 4000 rpm for 15 minutes and the supernatant was combined with Salkowski reagent (0.5 M $FeCl_3$ and 35% perchloric acid), at a ratio of one milliliter to two milliliters ($HClO_4$). For 30 minutes, this solution was kept at room temperature and the emergence of a pink hue indicated the presence of IAA.

2.1.10.8 Nitrate Reduction Test

In 100 ml of distilled water, 20 g peptone and 2 g potassium nitrate were mixed to make nitrate broth. Heavy inoculum of bacteria was transferred to media, aseptically. The test tubes were incubated at 35-37 °C for 4 hours and few drops of the reagent A (0.8 g sulfanilic acid in 70 ml of water) was added. The solution was heated to dissolve solvents and cooled. In the solution, 30 ml acetic acid was added and the solution was stored at 2 to 8 °C. In the final step, reagent B (30 ml of glacial acetic acid, 0.5 g of naphthylamine, 70

ml of distilled water) was added in the solution. The development of red color indicated the positive test.

2.1.10.9 Urease Activity

Urease activity solution was made by mixing 20 g urea (as a substrate), 0.01 g phenol red, 9 g KH_2PO_4, 0.1 g yeast extract, and 9.25 g Na_2HPO_4 in 1000 ml of distilled water. To assess urease activity, 5 ml of broth media (pH 8.4) was combined with the prepared solution. Each bacterial strain was tested in two test tubes, with one served as control. Excluding control, all tubes were contaminated with bacterial cultures. The infected tubes were kept at 30 °C for one day before being transferred to 37 °C, for a week. A positive test for urease activity observed when the phenol red indicator developed a pink tint at a pH greater than 8.4.

2.1.10.10 Siderophore Production

Bacteria were grown in LB broth culture, overnight and centrifuged to get supernatant. 10% O-CAS dye was added in LB agar medium. In a solidified media, using cork-borer, wells were made. In each of these wells, 100 mL supernatant of respective bacterial strain was poured. An orange hue that appears to surround the wells denoted successful production of sidérosphore.

2.1.10.11 Zinc Solubilization Assay

Selected bacterial strains were evaluated for zinc solubilization using tris-minimal salt media. This media was prepared by adding Tris HCl (6.06 g), D-glucose (10 g), KCl (1.49 g), NaCl (4.68 g), NH_4Cl (1.07 g), Na_2SO_4 (0.43 g), $CaCl_2.2H_2O$ (30 mg), $MgCl_2.2H_2O$ (0.2 g), and Agar (15 g). To test the solubilization of zinc oxide, ZnO (0.1%) was added to the media as the only source of zinc. To find the clearing halo zone on agar media, the bacterial isolates were injected on the medium and incubated for seven days at 30-35 °C (Fasim *et al.,* 2002)

2.1.10.12 Hydrogen Cyanide Production

To measure the generation of hydrogen cyanide (HCN), rhizobacteria were spread out on LB agar containing 4.4 g/L glycine. Upper lid of each petri plate was then covered

with a filter paper strip that had been soaked in a solution (with 0.5% picric acid and 2% sodium carbonate). To prevent the gas emission, parafilm was properly wrapped around the petri plates. The color of filter papers was changed from yellow to orange brown after 2-3 days of incubation at 28 °C, indicating the production of hydrogen cyanide.

2.1.10.4 Amylase Activity

Starch agar media was prepared by adding 15 g agar, 3 g beef extract, 5 g peptic digest animal meat, and 2 g soluble starch in 1000 ml of distilled water. The pH of solution was maintained at 7.2. On the prepared media, a newly developed 24-hour-old bacterial colony was streaked and incubated for 24 hours at 37 °C. The plates were treated with an iodine solution. The development of a clear zone surrounding the growth line demonstrated amylase activity of bacteria.

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2.1.11 Growth Assay of Selected Bacterial Strains

Based on biochemical characterization and enzymatic activity analyses, three most efficient bacterial strains were selected, and their optimum growth were evaluated at variable pH (5-8) and temperature (28-42° C) conditions, in LB broth supplemented with 2% NaCl. While checking the effect of temperature on bacteria, the pH 7 was maintained by using concentrated sodium hydroxide (NaOH) and hydrochloric acid (HCl). The optical density of bacterial culture was determined at 660 nm, at regular time intervals to assess their growth.

2.1.12 Extraction and Purification of Phytohormones

For the detection and measurement of abscisic acid (ABA), gibberellic acid (GA3), and indole-3-acetic acid (IAA), three selected bacterial isolates were cultured in Pikovskaya's broth media (0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.0001 g $MnSO_4$, 0.2 g KCl), with or without tryptophan (0.01 g/100 ml). The mixture was placed in a shaking incubator at 100 rpm for 72 hours. The bacterial cultures were centrifuged for 15 minutes at 4 °C and 10,000 rpm. Method of Tien *et al*. (1979) was followed to separate supernatants and extract phytohormones. The samples were subsequently run using HPLC, and phytohormones were found using a standard retention time. IAA was examined at a wavelength of 280 nm whereas GA3 and ABA were

examined at a wavelength of 254 nm. The culture material that had not been inoculated with bacteria was used as blank.

2.1.13 Evaluation of PGPR under Salinity Stress in Wheat

Each selected bacterial isolate was inoculated in 100 ml LB broth and incubated at 37 °C and 120 rpm in a shaking incubator. After 48 hours, broth culture was centrifuged at 3000 rpm for 10 minutes. The resulting pellets were collected and suspended in distilled water to obtain desired optical density (OD=1) at 600 nm. Wheat seeds (Morocco variety) were collected from National Agricultural Research Centre *(*NARC*),* Islamabad and surface sterilized with 95% ethanol for 10 seconds and rinsed 5-6 times with autoclaved distilled water. Wheat seeds were bio-primed by soaking in different bacterial suspensions for 45 minutes and were air-dried prior sowing (Naseem and Bano, 2014). Seed priming was preferred because it is fast, cheap and accurate method and it requires low amount of inoculant and confers many other beneficial characteristics to the seeds (Mahmood *et al.,* 2016).

Seeds were sown and obtained seedlings were subjected to NaCl stress after every 3 days, for 3 weeks. On the alternate days, plants were watered normally. Some seeds were not primed with bacterial suspension and their seedlings served as control. Each treatment was replicated three times. The plants were placed in growth chamber at 12 h light/dark photoperiod with 20-22 °C (light) and 15-17 °C (dark) temperature. A constant 70% humidity was also maintained. After 21 days, plants were uprooted and their root length, shoot length and leaf areas were recorded. Later, wheat plants of different treatments were dried in an oven at 72 °C for 48 hours and grinded. These powder samples were digested by following wet digestion method of Enders and Lehmann (2012). Macro-nutrients (NPK) were quantified using Atomic Absorption Spectrophotometer (AAS).

2.1.14 Statistical Analysis

The experiments were carried out in triplicates and their mean and standard deviations were calculated using Excel 2016. The experiments were evaluated by one-way ANOVA and the means were assessed with Tukey's test at $p < 0.05$, using Statistix version 8.1.

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2.2 EXPERIMENT 2: HALOTOLERANT PLANT GROWTH PROMOTING RHIZOBACTERIA INDUCE SALINITY TOLERANCE IN WHEAT BY ENHANCING THE EXPRESSION OF SOS GENES

2.2.1 Collection of Rhizobacterial Strains

Three potential plant growth promoting rhizobacterial (PGPR) strains (*B. megaterium* MPP7, *B. tequilensis* MPP8 and *P. putida* MPP18) were used, in this study.

2.2.2 Bioassays of Isolated Bacterial Strains

2.2.2.1 ACC Deaminase Activity

Activity of 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) of all three bacterial strains was assessed by quantifying α-ketobutyrate. The bacterial strains were cultured in DF salt minimal medium supplemented with 0 mM, 25 mM, 50 mM, and 100 mM concentration of NaCl and 3 mM ACC. Cultured broth were placed in a shaking incubator at 30 ± 2 °C and 150 rpm for 1 day. The ACCD activity was assessed by following the procedure of Penrose and Glick (2003). Optical density values were recorded at 540 nm. A standard curve of α-ketobutyrate concentration was plotted against absorbance value of each tested sample and finally, the α-ketobutyrate was quantified in μM/mg protein/h.

2.2.2.2 Exopolysaccharide Production

To quantify the production of exopolysaccharide, the bacterial strains were cultured in ATCC no. 14 broth. This culture media was prepared by adding KH_2PO_4 (0.2 g), K_2HPO_4 (0.8 g), $CaSO_4.2H_2O$ (0.1 g), $Na_2MoO_4.2H_2O$ (10 mg), $MgSO_4.7H_2O$ (0.2 g), $FeCl_3$ (10 mg), yeast extract (0.5 g), agar (15 g) and sucrose (20 g) in distilled water (1000 ml). Before autoclaving, the pH of media was adjusted to 7.2. Bacterial strains were cultured and placed in shaker incubator for 48 hours at 30–35 °C and 200 rpm. The bacterial suspensions were centrifuged for 10 minutes at 9000 rpm after adding 1 mM EDTA to produce pellet. Supernatant containing exopolysaccharides and chilled acetone were mixed in 1:3 ratio and again centrifuged for 3 min at 15000 rpm to precipitate exopolysaccharides

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mass, as pellet. It was then washed with sterilized water and allowed to dry. The purified EPS was quantified gravimetrically using an analytical balance (Zainab *et al.,* 2020).

2.2.2.3 Synthesis of Indole Acetic Acid

IAA synthesis potential of selected three bacterial isolates was assessed by following the protocol of Gordon and Weber (1951). For this purpose, LB broth was prepared and supplemented with varying NaCl concentrations (i.e., 0, 25, 50, and 100 mM) and L-tryptophan (100 mg/L). The culture was incubated in shaker incubator at 35 °C ± 2 °C and 150 rpm for 1 day and each culture (5 ml) was centrifuged for 2 minutes at 15000 rpm. Supernatant and Salkowski's reagent were mixed in 1:2 ratios and placed in dark for 30 min. IAA production was checked by using Salkowski reagent. Color development was considered as an indicator of IAA production. The OD of solution (supernatant mixed with Salkowski's reagent) was measured at 535 nm. To calculate IAA production, standard curve was drawn with 10–100 µg/ml IAA.

2.2.2.4 Phosphate Solubilization

For the screening of phosphate solubilizing bacteria, plate assay method was used. Bacteria were allowed to grow on Pikoyskaya media, supplemented with various salt concentrations (i.e., 0, 25, 50, and 100 mM) and the Petri plates were placed in an incubator at 27–28 °C ± 2 °C for 7–8 days (Pikovskaya, 1948; Fischer *et al.,* 2007). After clear zone appearance around the bacterial strains, phosphate solubilization index was computed by the following formula:

$$PSI = Colony\ diameter + Halozone\ diameter \div colony\ diameter$$

2.2.3 Salt Tolerance Assay of Selected Bacterial Strains, In Vitro

To see salt tolerance, selected bacterial strains were separately inoculated into LB broth, amended with 0-100 mM concentration of NaCl (Barra *et al.,* 2016) in 100 ml flasks. The inoculated flasks were positioned in shaker incubator at 35 °C ± 2 °C for 1 day at 250 rpm. The growth of bacteria under saline conditions was observed for next seven days by measuring the OD of growth media at 600 nm.

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2.2.4 Salt Tolerance Assay of Selected Bacterial Strains, In Vivo

For this analysis, a pot experiment was carried out (in three replicates), in a complete randomized design (CRD). Seeds of wheat variety Morocco were obtained from National Agriculture Research Center (NARC), Islamabad, Pakistan. Bacterial suspensions were prepared in LB broth by placing inoculated media in incubator shaker at 35 °C ± 2 °C and 120 rpm. After two days, centrifugation of broth cultures was performed for 10 minutes at 3000 rpm. Later, the pellets were resuspended in distilled water to get an optical density (OD) of 1 at 600 nm. Bio-priming was performed by soaking certified wheat seeds (Morocco variety) in the selected bacterial suspensions (Naseem and Bano, 2014). A concoction of clay, sand and peat moss (1:1:1 ratio) was sieved through 2 mm mesh to remove soil micro-organisms and gravel. Following the standard protocols of McLean (1983), the pH and EC of the experimental soil was measured. In each treatment, salt stress was induced with various concentrations of sodium chloride (NaCl) (0 mM, 25 mM, 50 mM and 100 mM). About 200 g of autoclaved soil was added to each pot and seeds were sown in sets of the following treatments: soil containing non-primed (normal) seeds (C); soil containing seeds primed with *B. megaterium* (B1), soil containing seeds primed with *B. tequilensis* (B2) and soil containing seeds primed with *P. putida* (B3).

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2.2.4.1 Germination Percentage

Seeds were said to be germinated when 2 mm radical emerged from the seed coat. The germination percentage was recorded by using the following formula of Manmathan and Lapitan (2013):

$$\text{Germination percentage} = \text{germinated seeds} \div \text{total seeds} \times 100$$

After 21 days of sowing, following biochemical and physiological parameters were studied:

2.2.4.2 Growth Attributes

With the help of a measuring tape, the length of freshly harvested wheat shoots and roots were determined. Fresh weights of the plants were measured using electrical balance

after uprooting the whole plants. The weights of plants were measured after being oven dried for 72 hours at 70 °C.

2.2.4.3 Osmo-protectants

Procedure of Bates *et al*. (1973) was used to determine proline content in the leaves of wheat. To crush 0.1 g leaves, 4 ml aqueous solution of 3% sulfo salicylic acid was used. The mixtures were centrifuged at 3000 rpm for 5 minutes. In 2 ml of supernatant, 2 ml of acidic ninhydrin solution was added (made by dissolving 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid). The samples were then incubated in a water bath at 100 °C for 1 hour and allowed to cool. Absorbance was measured at a wavelength of 520 nm. The proline content was calculated using the following formula:

Proline Content = k × dilution factor × absorbance / sample weight

The methodology proposed by Hahm *et al*. (2017) was used to calculate the total soluble sugar content. In a glass tube containing 5 ml of warmed 80% (v/v) ethanol, 0.1 g of leaf tissue was homogenized. The aliquots of the homogenates were transferred to 2 ml centrifuge tubes and incubated at 80 °C for 30 minutes. The homogenates were then centrifuged for 10 minutes at 4 °C at 16,200 ×g and the supernatants were transferred in 1.5 ml Falcon tubes. A standard calibration curve ranging from 0 to 10 mg of carbohydrate sugar was used to quantify the total soluble sugar levels (µg/g FW) and determine the optical density (OD) at a wavelength of 620 nm.

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2.2.4.4 Photosynthetic Pigments

Freshly harvested leaves (0.1 g) were grinded in 80% acetone and kept in dark for 24 hours. For the determination of carotenoid and chlorophyll contents, absorbance of extracts was measured at variable pH (Stockburger and Mitchell, 1999). Photosynthetic pigments were calculated using below formulas:

Chl *a* = [12.7 (OD at 663 nm) – 2.69 (OD at 645 nm)] × V ÷ 1000 × W

Chl *b* = [22.9 (OD at 645 nm) – 4.68 (OD at 663 nm)] × V ÷ 1000 × W

$$\text{Carotenoids} = (\text{OD at } 480 \text{ nm}) \times 4$$

2.2.4.5 Relative Water Content

RWC was assessed by following the method of Whetherley (1950). Fresh leaf was taken and weighed as fresh weight (FW). This leaf was positioned in petri plate filled with distilled water, overnight, in dark. After 24 hours, the leaf turgid weight (TW) was determined by using sensitive weighing balance. The leaf was placed at 72 °C in an oven for overnight and the dry weight (DW) was determined. Leaf relative water content was evaluated using the following formula:

$$\text{RWC} (\%) = [(\text{FW} - \text{DW}) \div (\text{TW} - \text{DW})] \times 100$$

2.2.4.6 Relative electrolytic leakage

The leaf was sliced into small pieces (0.5 g) and placed in a test tube with 10 ml distilled water. Electric conductivity (R1) was measured using an electrical conductivity meter after the test tubes were kept at 4 °C overnight. The test tubes containing leaves were autoclaved for 30 min and the contents were allowed to cool (Lutts *et al.,* 1996). The electric conductivity was again determined (R2) using EC meter and noted.

Relative electrolytic leakage was determined:

$$\text{Relative Electrolytic leakage} (\%) = \text{R1} \div \text{R2} \times 100$$

2.2.4.7 Salt Tolerance Index

Fresh weight refers to the overall weight of these harvested plants. For 2 days at 72 °C, the pre-weighted plant was dried in an oven to assess biomass production. The following equation was used to determine the salt tolerance index (STI), as reported by (Shetty *et al.,* 1995).

$$\text{STI} = \text{BPS or BPI} \div \text{BPN} \times 100$$

Where BPS = biomass of plant under salt stress, BPI = biomass of inoculated plant, and BPN = biomass of non-stressed/uninoculated plants.

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2.2.4.8 Superoxide Dismutase (SOD) Assay

Plants were assessed for superoxide dismutase activity using the techniques of Beauchamp and Fridovich technique (1971).

For SOD activity analysis, following phosphate buffers were prepared:

(a) 15.6 g of monosodium dihydrogen phosphate was mixed with 500 ml of distilled water to make Monosodium Dihydrogen Phosphate Solution.

(b) 53.65 g of disodium hydrogen phosphate was mixed with 600 ml of distilled water to make Disodium Hydrogen Phosphate Solution.

97 ml of monosodium dihydrogen phosphate solution and 183 ml of disodium hydrogen phosphate solution were combined to make a pH 7 phosphate buffer, and the total volume was then increased to 600 ml by adding distilled water.

25.5 ml of monosodium dihydrogen phosphate solution and 275.5 ml of disodium hydrogen phosphate solution were combined to make a pH 7.8 phosphate buffer, and the total volume was then increased to 600 ml by adding distilled water.

For SOD activity analysis, following steps were followed:

(a) 1 g polyvinylpyrrolidone (PVP) and 0.0278 g Na_2EDTA were mixed and dissolved thoroughly in 100 ml phosphate buffer of pH 7.

(b) In an ice-cold pestle and mortar, around 0.2 g plant tissue was crushed in 4 ml of step (a) solution.

(c) The homogenized mixture was centrifuged at 4 °C for 10 minutes and the supernatant was separated. By adding phosphate buffer (pH 7) to the supernatant, the total volume was increased to 8 ml.

(d) Then, a phosphate buffer of pH 7.8 (100 ml) was mixed with 0.0278 g Na_2EDTA, 1.5 g methionine, and 0.04 g Nitro blue tetrazolium chloride (NBT).

(e) About 10 ml solution from step (d) was raised to 50 ml volume with phosphate buffer of pH 7.8.

(f) 0.0093 g of riboflavin was mixed in 100 ml phosphate buffer of pH 7.8.

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(g) By adding distilled water to a 20 ml solution from step f, the volume was increased to 50 ml.

Reference	Blank	Reaction Mixture
2 ml of step (e)	2 ml of step (e)	2 ml of step (e)
0.5 ml of step (g)	0.5 ml of step (g)	0.5 ml of step (g)
0.5 ml of enzyme step (c)	0.5 ml of pH 7 buffer	0.5 ml of enzyme step (c)

The reference samples were kept in full darkness during the experiment. The samples for reaction mixture, on the other hand, were kept in a light chamber for 20 minutes.

The absorbance at 560 nm was measured with a spectrophotometer. One unit of SOD activity is the amount of enzyme that, when compared to the control (which lacked enzyme), reduced the absorbance reading by 50%. Units/100 g F.W. were used to measure SOD activity. The final calculations were performed using the formula below:

R1- O.D of Reference, R2-O.D of Blank, R3- O.D of Sample

$$R4 = R3 - R2$$

$$A = R1\,(50/100)$$

$$Final = R4/A$$

2.2.4.9 Peroxidase (POD) Activity

The technique of Reddy *et al*. (1985) was used to assess peroxidase in plants, with minor changes.

<u>Preparation of 0.5 M Calcium Chloride Solution</u>

Calcium chloride (5.55 g) was placed in a graduated glass container and distilled water was added to a total volume of 100 ml. This solution was stored in a refrigerator and cooled on ice before use.

<u>Preparation of MES Buffer Solutions</u>

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MES buffer solution (0.02 M) was prepared by dissolving 293 mg in 75 ml of distilled water. The pH was changed to 6.0 with the addition of sodium hydroxide.

Preparation of 0.1 % p-*Phenylenediamine* (PPD)

To make this solution, 0.1 g p-*Phenylenediamine* (PPD) was suspended into 100 ml MES.

Crude Enzyme Extract Preparation

Following procedure was adopted to produce a crude enzyme extract:

1. Fresh or frozen plant material (1 g) was put in an ice-cold mortar and tissue was softened with a cool pestle with 5 ml of ice-cold calcium chloride solution (0.5 M).
2. Centrifugation of the mixture was done at 1000 rpm for 8 minutes. Supernatant was poured into a clean test tube and stored on ice.
3. The pellet in the centrifuge tube was resuspended in 2.5 ml of ice-cold calcium chloride solution and centrifuged again. The previously stored supernatant was merged with the newly collected supernatant.
4. Reaction mixture was prepared by mixing 0.1 ml enzyme extract + 1.5 ml MES + 0.5 ml (p-*Phenylenediamine*) + 0.45 ml H_2O_2, to a total volume of 2.55 ml (Approximately).

MES buffer was used as a blank and the reading was taken at 510 nm. After one reading (zero minute), the other was recorded after 3 minutes. Hydrogen peroxide was mixed in the reaction mixture right before placing the cuvette into spectrophotometer. This reading was recorded as zero-minute reading. Final reading was recorded after three minutes.

Based on these readings, following calculations were made:

Change in A510: $A_f - A_i$

A_f = Final reading (after 3 minutes)

A_i = Initial reading (zero minute)

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2.2.4.10 Catalase Activity

Catalase activity was evaluated by using the technique of Luck (1974).

Preparation of Buffer Solution (pH 7)

Phosphate buffer (0.067 M) was prepared by adding 5.963 g disodium hydrogen phosphate and 5.226 g mono sodium di hydrogen phosphate in 500 ml of distilled water. The pH was maintained at 7.

H_2O_2 solution

To prepare 2 Mm H_2O_2 solution, 12.6 µl of H_2O_2 was mixed with 100 ml of phosphate buffer (pH 7).

Procedure

Plant material (0.5 g) was grinded in 8 ml of phosphate buffer (pH 7) and centrifuged for 10 minutes and supernatant was collected. Now, 40 ml of supernatant was combined with 3 ml of 2 mM H_2O_2 and at 250 nm, its absorbance was measured using a spectrophotometer.

2.2.4.11 Oxidative Burst

The thiobarbituric acid (TBA) test, which detects MDA as an end product of lipid peroxidation was employed to assess lipid peroxidation in leaves. In 5 ml of 0.1 % (w/v) TCA solution, an aliquot (0.07 g) of leaves was homogenized. After centrifugation of the homogenate at 12,000 g for 15 minutes, 0.5 ml of the supernatant was added to 1 ml of 0.5 % (w/v) TBA in 20% TCA and incubated for 30 minutes boiling water. The reaction was halted by immersing the reaction tubes in an ice bath. The samples were then centrifuged for 5 minutes at 10,000 g, and the absorbance of the supernatant was measured at 532 nm after deducting non-specific absorption value at 600 nm. Malondialdehyde equivalents were determined by the following formula (Du and Bramlage, 1992; Munis *et al.,* 2010):

$$MDA = 6.45 (A_{532} – A_{600}) – 0.56 A_{440}.$$

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The production of H_2O_2 was assessed by mixing 0.2 g of leaf sample in 5 ml of 0.1% chilled trichloroacetic acid (Loreto and Velikova, 2001). At 390 nm wavelength, the absorbance was measured.

2.2.5 Expression Analysis of Stress Related Genes

The total RNA of control, salinity stressed and PGPR (*B. tequilensis*) inoculated wheat plants was extracted by CTAB method (Yu *et al.,* 2017). Quantitative real time PCR was performed for the expression analysis of selected salt stress related genes (SOS1 and SOS4), using forward and reverse primers (Table 2.1). Actin was used as a housekeeping gene. Three replicates were taken from each treatment. Protocol of Ho Kim *et al*. (2008) was used for the preparation of PCR mixture. Standard thermal cycling conditions were 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 seconds, 57 °C for 15 seconds and 72 °C for 45 seconds.

2.2.6 Statistical Analysis

The experiments were carried out in triplicates and their mean and standard errors were calculated using Excel 2016. The data was subjected to one-way ANOVA, followed by Tukey's least significant difference (LSD) method using Statistix version 8.1. Furthermore, Principal Component Analysis (PCA) was performed by using XLSTAT 2016, to compare different experimental treatments.

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Table 2.1: Primer sequences of selected genes for real time PCR.

Gene name	Primer	Sequence	Reference
SOS1	Forward	5'- GTTGTCGGTGAGGTCGGAGGG -3'	Ramezani *et al*. 2013
	Reverse	5'- TCATCTTCTCCTACCGCCCTGC-3'	
SOS4	Forward	5'-ATCCAGTCCCACACCGTCCA -3'	
	Reverse	5'- GCTGATTGCCATTGAGAACCTGTC-3'	

2.3 EXPERIMENT 3: BIOFILM FORMATION AND FLOCCULATION POTENTIAL ANALYSIS OF HALOTOLERANT *BACILLUS TEQUILENSIS* AND ITS INOCULATION IN SOIL TO MITIGATE SALINITY STRESS OF CHICKPEA

2.3.1 Selection of PGPR

For the estimation of flocculation yield potential and biofilm formation ability, available strain of *Bacillus tequilensis* was selected. In the previous experiment, we have already reported the capability of this PGPR in phosphorus solubilization and the production of indole acetic acid, siderophore, HCN, EPS, and ACC-deaminase.

2.3.2 Estimation of Bacterial Flocculation

To make Tryptic soy broth (TSB) media, 3 g of Soy, 17 g of Tryptone, 5 g of NaCl, 2.5 g of glucose and 2.5 g of dipotassium phosphate (K_2HPO_4) were suspended in 1000 ml of distilled water and autoclaved. *B. tequilensis* was cultured in TSB broth and incubated at 30-35 °C for 4 days. With the help of Whatman filter paper No. 1, the culture was filtered, and the collected flocculation was dried by placing it into an oven for 2 hours at 60 °C. The dry weight was recorded and presented as floc yield (Sadasivan and Neyra, 1985).

2.3.3 Biofilm Formation

The microtiter plate-based protocol was used to estimate biofilm formation. For 24 hours, *B. tequilensis* was cultured in TSB medium, amended with NaCl at 30-35 °C and its optical density (OD) was observed using UV visible spectrophotometer (752N UV-VIS, Beijing, China) and adjusted to 0.3. The bacterial culture (200 ml) was transferred into the wells of microtiter plate and incubated at 35-37 °C. After 4 days, the growing media was removed, and the wells were stained for 20-25 minutes with 0.01 % crystal violet. The stained biofilm, formed on the walls of microtiter plate wells was extracted with 95% ethanol and its OD was recorded at 590 nm (Christensen *et al.,* 1985).

2.3.4 Scanning Electron Microscopy (SEM) of B. tequilensis Under Salinity Stress

B. tequilensis was grown in TSB medium, modified with 100 mM NaCl and shaken for 4 days at 120 rpm in an incubator shaker. The bacterial pellet was obtained after centrifuging the culture for 10 minutes at 5000 rpm. The bacterial cells were treated with

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2.5 % glutaraldehyde at 4 ˚C for 5-6 hours and then centrifuged for 6-8 minutes at 5000 rpm. The pellet was rinsed using 0.1 M sodium cacodylate buffer for 10 minutes. The sample was treated with 1% osmium tetraoxide for post-fixation and dehydrated with 35-100 % acetone. In a critical dryer, the sample was dried for 1-2 hours. The dried sample was placed on a stub and sputter coated with gold before being examined under a scanning electron microscope (SEM, JEOLJSM 25910).

2.3.5 Fourier Transform Infrared Spectroscopy (FTIR)

In a previous study, we found that *B. tequilensis* could produce exopolysaccharides (EPS) under varying levels of salt stress. To characterize EPS in this study, two milligrams of the extracted dried EPS from *B. tequilensis* was mixed with 200 mg of potassium bromide and subjected to FTIR spectroscopy. Functional groups of EPS were determined in a range of 4000–5000 cm^{-1} (Model No. FTSW 300 MX, BIO-RAD, California, USA).

2.3.6 Screening of Genes Conferring PGP Traits

Two most important plant growth promoting (PGP) genes of bacterium were detected by their amplification with conventional PCR. DNA of *B. tequilensis* (PCR template) was isolated (William *et al.,* 2012) and gene specific primers (Table 2.2) were used to amplify 1-aminocyclopropane-1-carboxylate deaminase (acdS) and pyrroloquinoline quinone (pqqE) genes. acdS gene enhances plant growth by reducing ethylene levels (Naing *et al.,* 2021), while pqqE gene encodes PPQ cofactor for phosphate solubilization (Kim *et al.,* 2003).

Table 2.2: Primer sequences of selected genes for RT-PCR.

Gene name	Primer	Sequence
AcdS	Forward	5'- ATGAAYCTSCARCGHTTY -3'
	Reverse	5'- TYARCCGTYSCGRAARRT -3'
PqqE	Forward	5'- GARCTGACYTAYCGCTGYCC -3'
	Reverse	5'- TSAGSAKRARSGCCTGR -3'

2.3.7 Pot Experiment

2.3.7.1 Inoculation and Sowing of Chickpea Seeds

Seeds of Kabuli chickpea variety (Punjab 2008) were obtained from National

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agricultural research center (NARC), Islamabad. Seeds were rinsed with tap water and immersed in 1 % sodium hypochlorite (NaOCl) solution for 3 minutes and then again washed three times with deionized water. Seeds were sterilized and dried on filter paper before being nicked with a nail clipper. Chickpea seeds were inoculated by soaking in suspension of bacterial culture for 1 hour. The seeds were then air dried aseptically in a laminar air flow. Surface sterilized control seeds were submerged in sterilized distilled water only.

Seeds were planted in plastic pots and placed in a controlled environment in the growth chamber at 20-25 °C, 60% relative humidity, and a light/dark cycle of 14/10 hr. Seven seeds were planted in each pot, with three replicates for each treatment (Table 2.3). For three weeks, seedlings were subjected to saline water, every third day. Pots with control plants were irrigated with normal water.

Table 2.3: Experimental treatments used in pot experiment.

Salinity level	**Treatment No.**	**Seed Treatment**
Control (0 mM NaCl)	Treatment 1 (T1) Treatment 2 (T2)	Uninoculated seeds *B. tequilensis* inoculated seeds
Saline (25 mM NaCl)	Treatment 3 (T3) Treatment 4 (T4)	Uninoculated seeds *B. tequilensis* inoculated seeds
Saline (50 mM NaCl)	Treatment 5 (T5) Treatment 6 (T6)	Uninoculated seeds *B. tequilensis* inoculated seeds
Saline (100 mM NaCl)	Treatment 7 (T7) Treatment 8 (T8)	Uninoculated seeds *B. tequilensis* inoculated seeds

2.3.7.2 Germination Rate

Following the protocol of Manmathan and Lapitan (2013), germination rate was calculated in percentage after 2 mm emergence of radical from the seed.

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2.3.7.3 Plant Analysis

Chickpea seedlings were collected three weeks after planting and their root and shoot lengths were calculated. The fresh weight of plants in each treatment was recorded. Relative electrolyte leakage (REL) of chickpea seedlings was measured in deionized water at 40 °C and 100 °C (Sairam *et al*., 2002). The relative water content (RWC) of freshly harvested chickpea leaves were calculated using Whetherley's formula (1950).

The seedling leaves were ground in acetone and centrifuged. Using spectrophotometer, absorbance of supernatant was calculated to determine chlorophyll (Porra, 2002) and carotenoid contents (Lichtenthaler and Wellburn, 1983).

For the determination of proline content, method of Bates *et al.* (1973) was used. The methodology of Hahm *et al.* (2017) was used to estimate total soluble sugar content in leaves of chickpea seedlings. Using the tests developed by Haroon *et al*. (2021), antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) were evaluated in fresh chickpea leaves. Salt tolerance index (STI) of each seedling was calculated as the ratio of the value for the NaCl-treated seedlings to the value of the control seedlings.

2.3.8 Statistical Analysis

With three replicates, the experiments were designed in a completely randomized design (CRD) factorial. The database of parameters and results was created in MS Excel. Statistix 8.1 was employed to perform analysis of variance (ANOVA) on the collected data. The statistical significance of treatment mean values was determined using the HSD value of $p<0.05$. Principal component analysis (PCA) correlation was performed utilizing XL-STAT 2021. RStudio was used to create scatter plots.

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2.4 EXPERIMENT 4: LINKING SODIUM ACCUMULATION WITH SALT INDUCED EXPRESSION OF INTRACELLULAR VESICLE TRAFFICKING GENES WITH SALINITY STRESS TOLERANCE STRATEGY INDUCED BY *BACILLUS TEQUILENSIS* IN CHICKPEA

2.4.1 Bio-priming of Disinfected Seeds

Viable seeds of chickpea variety (Punjab-2008) were disinfected with 0.01% sodium hypochloride solution and washed at least three times with distilled water. The halotolerant *B. tequilensis* was chosen and cultured in LB broth (Haroon *et al.,* 2021). With the help of a nail clipper, seeds were nicked and treated with bacterial culture by dipping them for three hours. Control seeds were immersed in distilled water and shade dried before sowing.

2.4.2 Experimental Design and Treatment Pattern

In March 2021, normal and treated seeds were sown in pots with sandy loam soil and manure and placed in a glass house in their natural state. Seedlings were stressed with NaCl (100 mM) every three days for three weeks. On alternate days, the plants were watered as usual. The experiment was done in triplicates in a randomized complete block design. Plants were sown in three different experimental treatments (Table 2.4).

Table 2.4: Experimental treatments used in pot experiment.

Treatment Code	Description
C	Plant without the influence of salinity and *PGPR*
S	Plants under the influence of 100 mM NaCl stress
S + PGPR	Plants under the influence of 100 mM NaCl and *B. tequilensis*

2.4.3 Gas Exchange Measurements

Using an infrared gas analyzer, intercellular CO_2 concentration (Ci), Net CO_2 assimilation (A), transpiration rate (Tr), stomatal conductance g(s), leaf temperature and leaf vapor pressure deficit (VPD), were recorded from third completely grown leaves, 40 days after salt treatment (Li-Cor Inc., Lincoln, NE, USA). In the measuring chamber, 400

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mol mol^{-1} CO_2 level, 65% relative humidity, 500 mol s^{-1} flow rate, and 1500 mol m^{-2} s^{-1} saturating PAR was maintained. Gas exchange measurements were obtained during full daylight for maximum photosynthesis (about 10 a.m. to 12 p.m.). To reduce the impact of time on gas exchange measurements, plants in each replication were measured at random.

2.4.4 Stomatal Assay

Transparent nail polish and sticky tape were used to capture imprints of the abaxial epidermis at the location of leaf area width close to the main vein of each treatment's third completely grown leaf. At a magnification of 400×, all the impressions were inspected under a light microscope and three tiny areas per replica were chosen, at random. A digital camera was used to take microphotographs of each location (Olympus C-4040 ZOOM, Japan). Image J was used to process all photos (NIH, USA). Stomatal length, stomatal width, pore length and pore width were measured and expressed in centimeters (cm).

2.4.5 Leaf Injury Index

The extent of leaf necrosis and abscission was used to quantify salt injury to plants on a regular basis (qualitative observations). The degree of leaf damage and the percentage of live plants were measured and rated after 40 days of salt stress. As previously indicated, based on 5 level scales (Table 2.5), depending on the level of necrotic tissues and the quantity of damaged plants, the injury index was computed (Zhen *et al.,* 2010).

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Table 2.5: Five level scale to classify plants on the percentage of necrotic tissue.

Level	Percentage of necrotic area
1	0%
2	1–25%
3	26–50%
4	51–75%
5	76–100%

Salt injury index was determined by multiplying the level of injury with the total number of injured plants and by dividing it with the total number of plants in a particular treatment.

2.4.6 Leaf Elemental Content and Osmolality

After 40 days of NaCl treatment, the youngest completely developed leaf was removed (three replicates from each treatment). To extract sap, leaves were squeezed by hand in Eppendorf tubes and centrifuged at 7000 g for 10 minutes; A vapour pressure osmometer was used to assess the osmolality of 20 mL of recovered supernatant (Vapro, Wescor Inc., Logan, UT, USA). In addition, the quantities of sodium and potassium ions in the leaf sap were determined by mixing 50 mL of the retrieved supernatant with 5 mL of distilled water and measuring the mixture in a flame photometer (Corning 410C, Essex, UK).

2.4.7 PCR Analysis

Total RNA was isolated from control, salinity stressed, and PGPR (*B. tequilensis*) injected chickpea plants using the Qiagen RNeasy Kit, as directed by the manufacturer. Semi-quantitative RT-PCR and qRT-PCR studies were performed on cDNA samples diluted in water (1:10). cDNA from S+PGPR was utilized as a template in semi-quantitative RT-PCR. Reaction mixture was prepared by mixing 1.8 mM MgCl2, 250 mM of each forward and reverse primers, 0.2 mM dNTP, and 1.0 unit of Go-Taq DNA polymerase. The following program was used for amplification: 94°C for 2 minutes; 30 cycles of 94°C for 10 seconds, 55°C for 10 seconds, and 72°C for 15 seconds; and a final extension at 72°C for 1 minute. The GelDoc was used to visualize the gel contain GelRed. Amplicon sizes ranged from 88 to 210 bp, and primer information may be found in Table 2.6.

KAPA SYBR-Fast qPCR Universal ReadyMix was used for the quantitative reverse-transcriptase PCR (qRT-PCR). The Ready mix contained 0.1 µM of gene-specific primer sets as detailed below, in a final assay volume of 10 µl. Thermal cycling settings included a 3-minute initial melt at 95 °C, 40 cycles of 95 °C (5 s) and 60–61 °C (20 s), and a melt curve from 60 to 95 °C increasing by 0.5 °C increments every 5 s. A C1000

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thermocycler with a real-time PCR detection system was used to load samples (BioRad, CA).

2.4.8 Statistical Analysis

Means, standard errors, correlation and regression functions were calculated and analyzed using Microsoft Excel software. Using Statistix version 8.1, the data was treated to one-way ANOVA, followed by Tukey's least significant difference (HSD) procedure. Correlogram was constructed using Rstudio software.

Table 2.6: Primer sequences of selected genes for real time PCR (Sweetman *et al*., 2020).

Gene name	Primer	Sequence	Amplicon size
CaRabA2	Forward	5'- TTCBAACATTGTKATYATGATG -3'	210
	Reverse	5'- TGDGCWGCAAGTGCTTTTTAC -3'	
CaRabB	Forward	5'- TCAYTTRGCWAGYTGGTTGGAAGA -3'	194
	Reverse	5'-AWGCCTCTTCWACRTTYTGAGC -3'	
CaRabC	Forward	5'- GTTAAARCTTRCYATTTGGGA-3'	88
	Reverse	5'- AATTATTCCTTGTGCWCCTC-3'	
CaRabD	Forward	5'- TGAAATTGACCGHTATGCMAGT-3'	146
	Reverse	5'- TWGCACTTGTYTCCATRAAAGG-3'	
CaRabE	Forward	5'- GTGGWGCYATGGGHATHTTGC-3'	165
	Reverse	5'- TWGGHACAGCCCTTTTVCTTTC-3'	
CaRabH	Forward	5'- ACAARCTSGTTTTCTTAGGYGATC-3'	167
	Reverse	5'- CDGTATCCCAHARCTGCAGHCG-3'	

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3. RESULTS

3.1 EXPERIMENT 1

3.1.1 Physiochemical and Nutrient Analysis of Soil

Analyses of rhizospheric soil samples revealed a specific range of pH (7.5 to 7.9) and EC values (2.20 mS/m to 2.60 mS/m). Among the collected soil samples, IS1 exhibited the maximum value of EC (2.60 mS/m) while the minimum EC value (2.20 mS/m) was observed in IS2 sample. The texture of soil was found to be sandy loam and it was containing organic matter, nitrogen, phosphorus and potassium content, in a reasonable amount (Table 3.1).

Table 3.1: Basic properties and nutrient analysis of collected soil samples.

Sample No.	**Soil Properties**						
	pH	***Electrical conductivity (ms/m)***	***Soil texture***	***Organic matter (%)***	***Nitrogen (%)***	***Phosphorus (%)***	***Potassium (%)***
IS1	7.9	2.60	Sandy loam	1.57	0.079	0.152	0.57
IS2	7.5	2.20	Sandy loam	1.69	0.092	0.177	0.70
IS3	7.7	2.35	Sandy loam	1.62	0.084	0.163	0.64

3.1.2 Salt Tolerance Ability of Selected Bacterial Strains

At 2% NaCl concentration, 20 bacterial strains were isolated, successfully (Table 3.2). Out of these, six bacterial isolates (MPP1, MPP7, MPP8, MPP12, MPP15 and MPP18) were able to tolerate and grow at 10% concentration of NaCl. Four strains (MPP4, MPP5, MPP13 and MPP20) could tolerate up to 8% NaCl concentration, while three bacterial strains (MPP9, MPP14 and MPP19) were unable to grow at more than 2% NaCl

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concentration. Six bacterial strains, having the ability to tolerate maximum concentration of NaCl were selected for further analysis.

Table 3.2: Salt tolerance ability of isolated bacterial strains.

Bacterial isolates	**Concentration of NaCl**				
	2%	***4%***	***6%***	***8%***	***10%***
MPP1	+	+	+	+	+
MPP2	+	+	+	-	-
MPP3	+	+	-	-	-
MPP4	+	+	+	+	-
MPP5	+	+	+	+	-
MPP6	+	+	+	-	-
MPP7	+	+	+	+	+
MPP8	+	+	+	+	+
MPP9	+	-	-	-	-
MPP10	+	+	-	-	-
MPP11	+	+	+	-	-
MPP12	+	+	+	+	+
MPP13	+	+	+	+	-
MPP14	+	-	-	-	-
MPP15	+	+	+	+	+
MPP16	+	+	-	-	-
MPP17	+	+	-	-	-
MPP18	+	+	+	+	+
MPP20	+	+	+	+	-

'+' tolerable '-' not tolerable

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3.1.3 Morphological and Microscopic Characteristics

Different morphological and microscopic studies efficiently helped us to characterize selected bacterial strains (Fig. 3.1, 3.2 and Table 3.3).

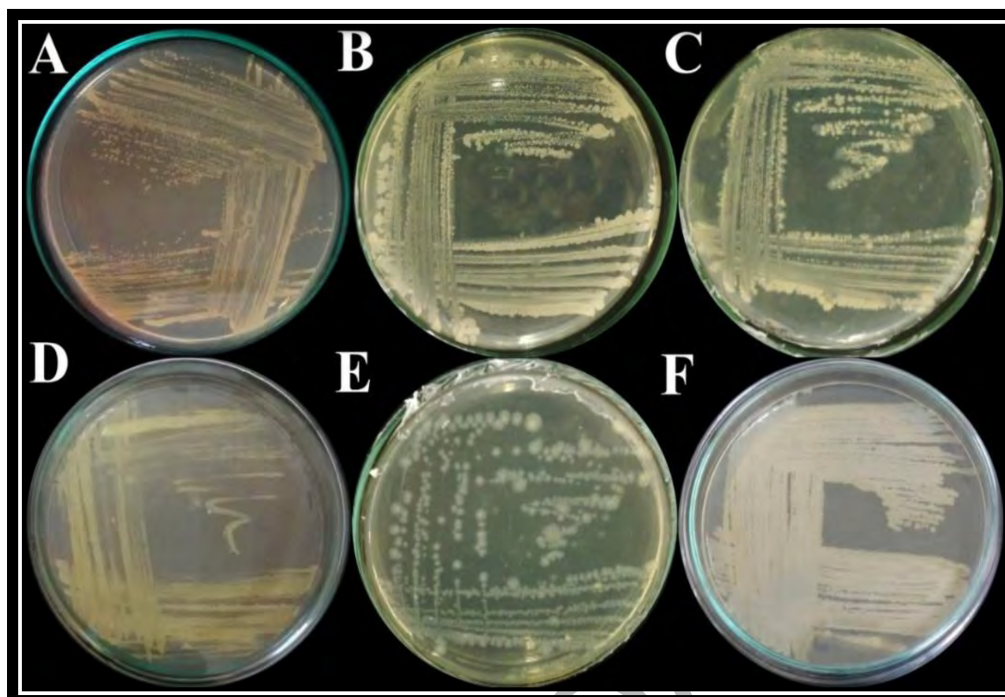

Fig. 3.1 Growth of selected halotolerant bacterial strains on LB media. (A) MPP1, (B) MPP7, (C) MPP8, (D) MPP12, (E) MPP15 and (F) MPP18.

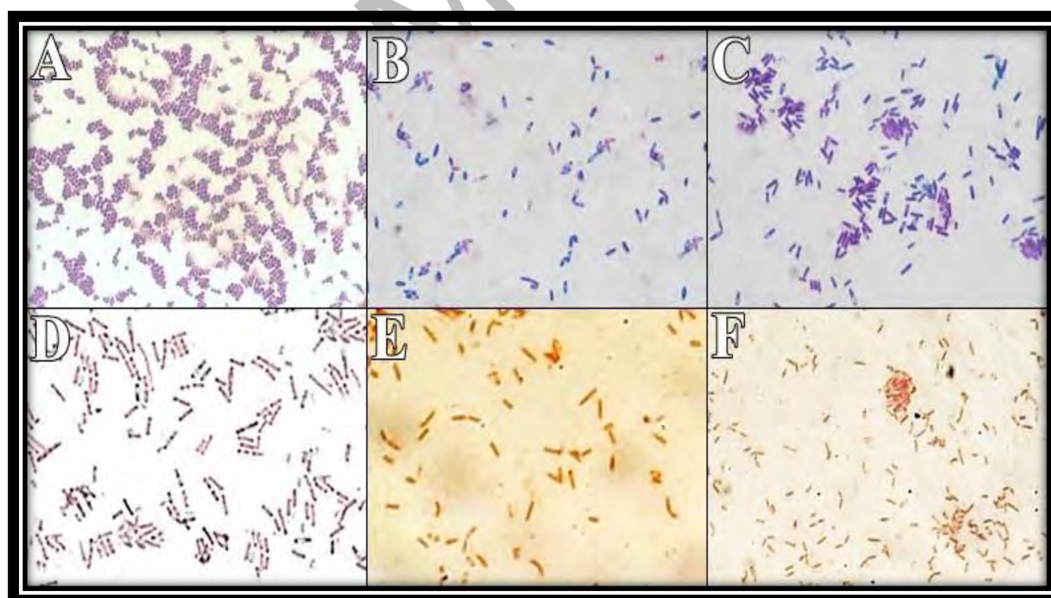

Fig. 3.2 Microscopic identification of selected halotolerant bacterial strains. (A) MPP1, (B) MPP7, (C) MPP8, (D) MPP12, (E) MPP15 and (F) MPP18.

Table 3.3: Colony morphology and microscopic observation of selected bacterial strains.

Morphological characteristics	**MPP1**	**MPP7**	**MPP8**	**MPP12**	**MPP15**	**MPP18**
Colony morphology	Yellow and raised colonies	Yellow, round colonies with irregular margins	Yellowish, smooth and circular colonies	Slightly, yellow, rough circular colonies	Large light yellowish flat colonies	Diffusible light-yellow colonies
Consistency	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Gram	+	+	+	+	-	-
Shape	Cocci	Rod	Rod	Rod	Rod	Rod
Motility	-	+	+	+	+	+

3.1.4 Molecular Characterization and Phylogenetic Analysis

Obtained FASTA sequences of all the selected bacterial strains have been presented in Appendix 1. Alignments of 16S rRNA gene sequences revealed similarities of the six selected isolates with *Bacillus megaterium, Bacillus tequilensis, Bacillus xiamenensis, Pseudomonas putida, Pseudomonas aeruginosa* and *Staphylococcus pasteuri* (Table 3.4). The phylogenetic trees successfully described evolutionary relationships of these bacteria (Fig. 3.3).

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Table 3.4: Molecular identification of six selected bacterial isolates.

Isolate code	**Accession no.**	**Nearest strain**	**Sequence length**	**Sequence identity (%)**
MPP1	MW237672	*Staphylococcus pasteuri*	1474	99%
MPP7	MW301077	*Bacillus megaterium*	1444	98.97%
MPP8	MW301075	*Bacillus tequilensis*	1394	98.8%

Isolate code	Accession no.	Nearest strain	Sequence length	Sequence identity (%)
MPP12	MW301076	*Bacillus xiamenensis*	1478	98.80%
MPP15	MW237670	*Pseudomonas aeruginosa*	1045	100%
MPP18	MW237671	*Pseudomonas putida*	1282	100%

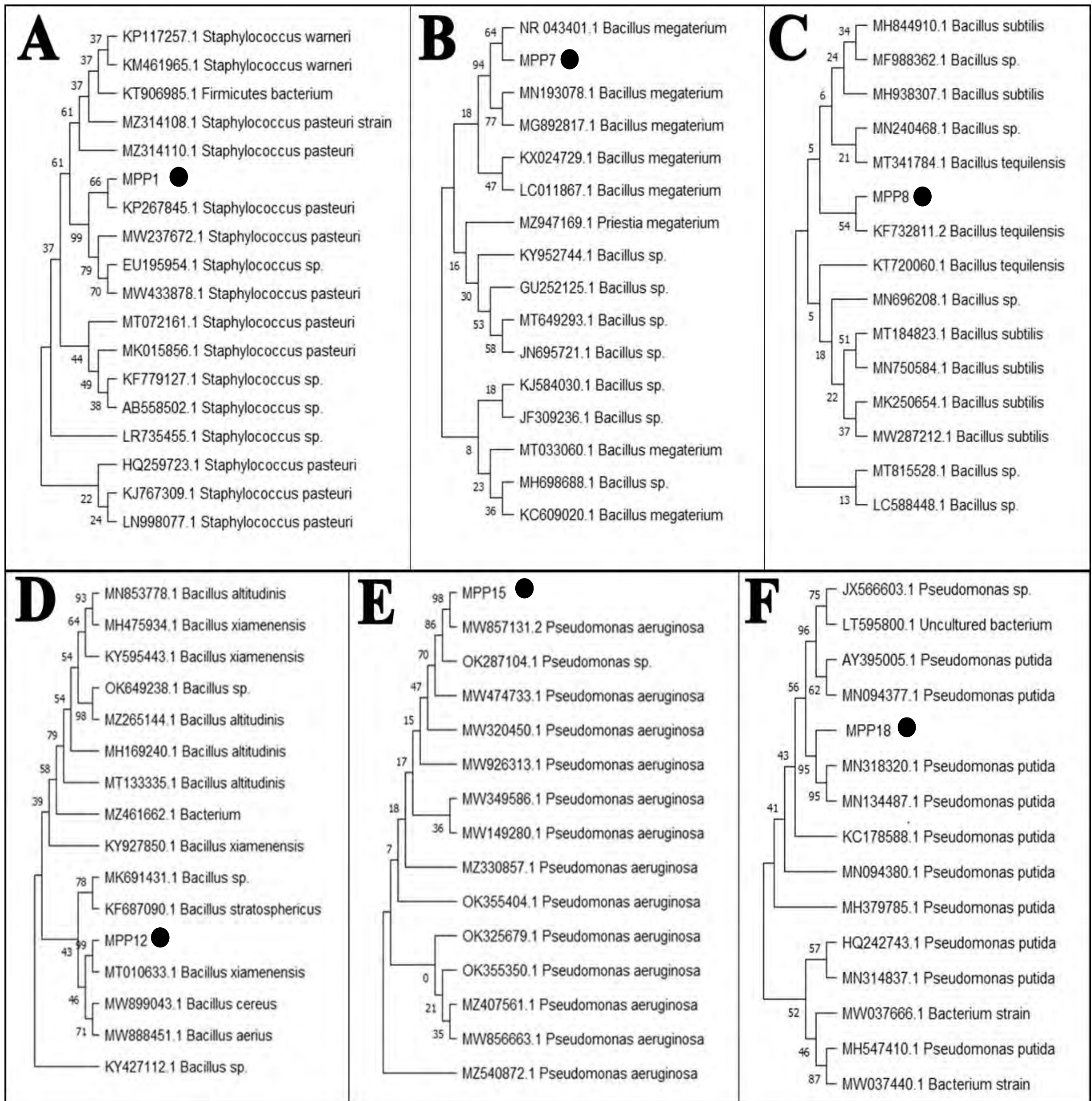


Fig. 3.3 Phylogenetic trees of all the six selected bacterial strains.

3.1.5 Antibiotic Resistance

All six selected isolates showed variable resistance against a variety of antibiotics (Fig. 3.4). Among these, *B. tequilensis* (MPP8) exhibited maximum tolerance to a variety of antibiotics, while *B. megaterium* (MPP7) also displayed considerable antibiotic resistance (Table 3.5).

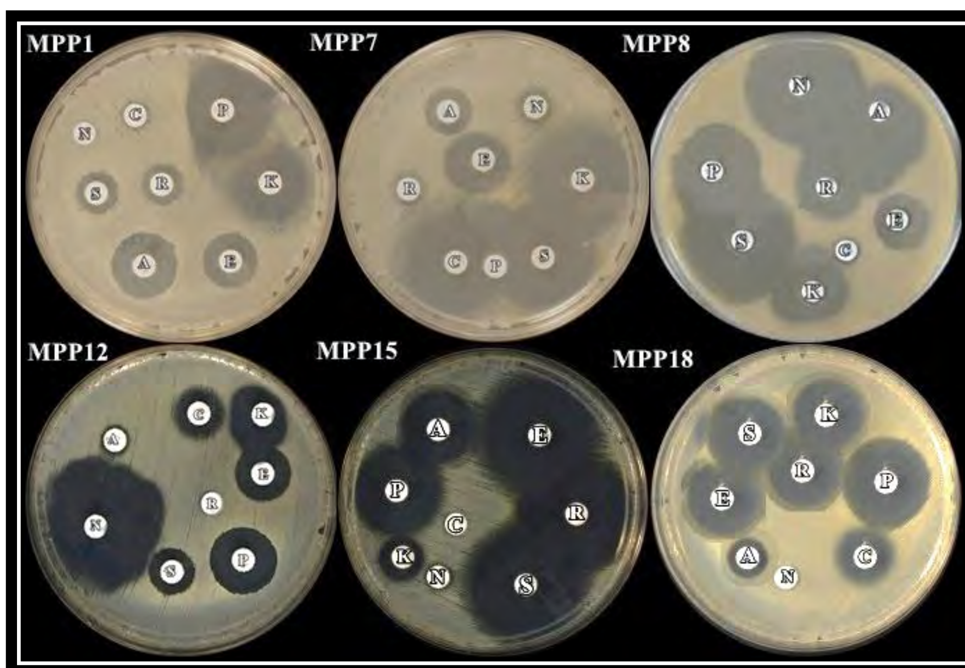


Fig. 3.4 Antibiotic resistance activity of selected bacterial strains against 8 different antibiotics including N (Neomycin), P (Penicillin), S (Streptomycin), K (Kanamycin), R (Rifampicin), A (Ampicillin), C (Chloramphenicol) and E (Erythromycin). Zone of inhibition was measured after 24 hrs of incubation.

Table 3.5: Antibiotics sensitivity tests of selected bacterial strains.

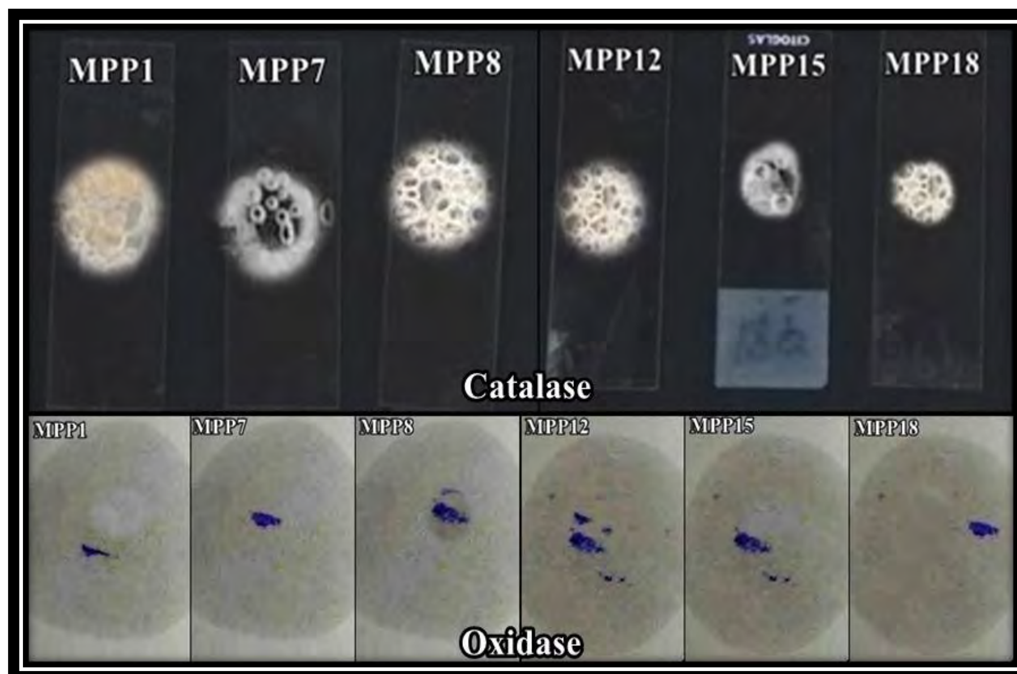
Antibiotics	Isolates with level of sensitivity					
	MPP1	*MPP7*	*MPP8*	*MPP12*	*MPP15*	*MPP18*
Neomycin	S	I	R	R	S	S
Penicillin	R	R	R	R	R	R
Streptomycin	I	R	R	I	R	R
Kanamycin	R	R	R	R	I	R

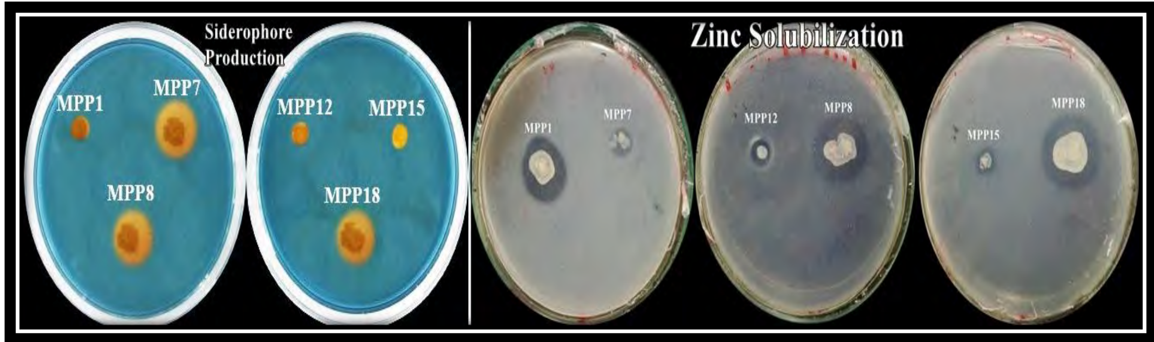
Antibiotics	Isolates with level of sensitivity					
Rifampicin	***MPP1***	***MPP7***	***MPP8***	***MPP12***	***MPP15***	***MPP18***
Ampicillin	R	I	R	S	R	I
Chloramphenicol	S	R	S	I	S	I
Erythromycin	R	R	I	R	R	R

'S' Sensitive; 'I' Intermediate; 'R' Resistance

3.1.6 Plant Growth Promoting Activity Assay

Study of various enzymatic activities helped us to understand the potential of selected isolates in growth promoting activities (Fig. 3.5). As shown in Table 3.6, the comprehensive findings revealed maximum enzymatic activities of *B. tequilensis* (MPP8).




Siderophore Production
MPP1
MPP7
MPP8
MPP12
MPP15
MPP18
Zinc Solubilization
MPP1
MPP7
MPP12
MPP8
MPP15
MPP18

IAA Production
MPP1
MPP7
MPP8
MPP12
MPP15
MPP18
Nitrate Reduction
MPP1
MPP7
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MPP18
Urease
MPP1
MPP7
MPP8
MPP12
MPP15
MPP18

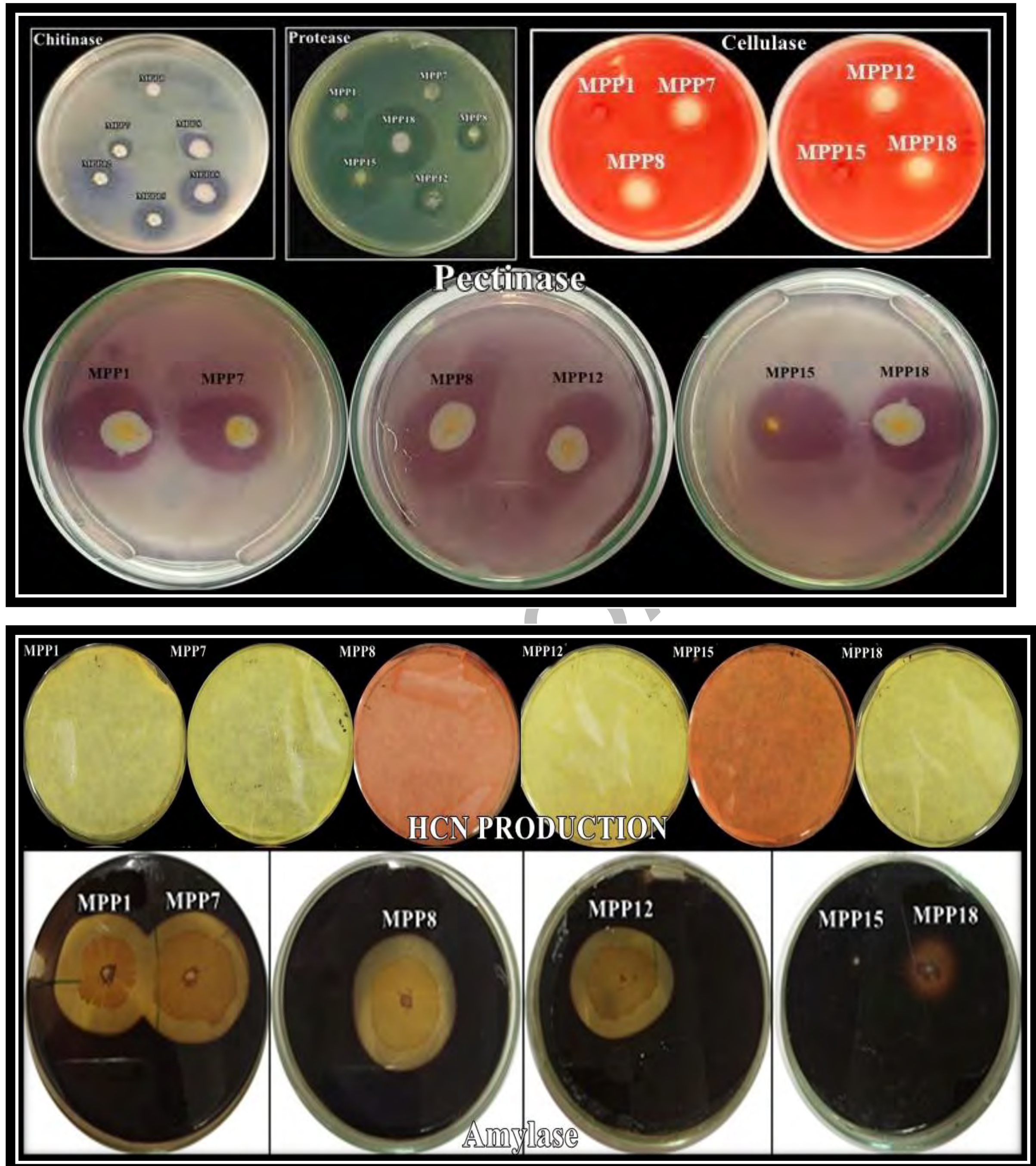

Fig. 3.5 Various plant growth promoting activities of selected halotolerant bacterial strains. Tested strains showed variable results for catalase, oxidase, siderophore production, zinc solubilization, IAA, production, nitrate reduction test, urease, chitinase, pectinase, cellulose, pectinase, HCN production and amylase production.

Table 3.6: Enzymatic activities and biochemical characterization of selected bacterial strains.

Biochemical Tests	MPP1	MPP7	MPP8	MPP12	MPP15	MPP18
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Chitinase	-	+	+	+	+	+
Protease	+	-	+	+	+	+
Cellulase	-	+	+	+	-	+
Pectinase	+	+	+	+	-	+
IAA production	-	+	+	+	-	+
Nitrate reduction	-	+	+	-	+	-
Urease	+	+	-	+	-	-
Siderophore production	-	+	+	-	-	+
Zinc solubilization	+	+	+	+	+	+
HCN production	-	-	+	-	-	+
Amylase	+	+	+	+	-	+

'+' presence '-' absence

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3.1.7 Analysis of Phytohormones

After the determination of PGP activities, three most efficient bacterial strains were selected and they all showed the ability to secrete IAAs, ABA and GA3, in the presence and absence of tryptophan (Fig. 3.6). Addition of tryptophan enhanced the production of

IAA while the concentration of ABA and GA3 were decreased by the addition of tryptophan.

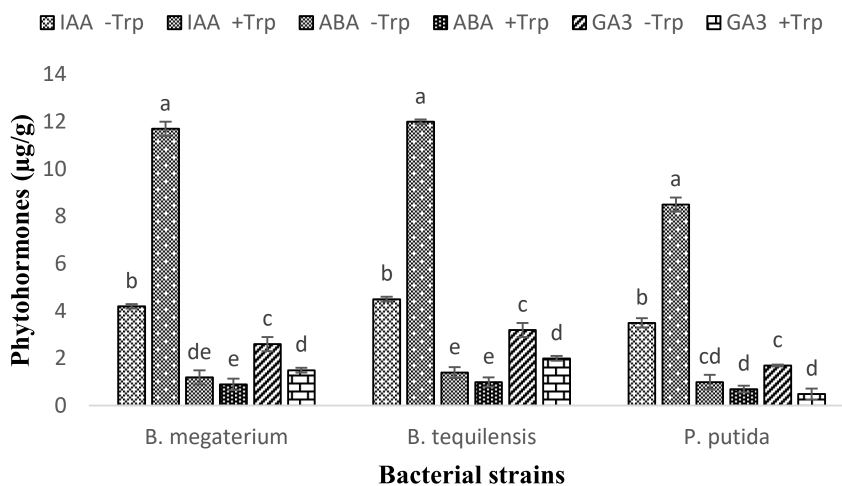

Fig. 3.6 The production of phytohormones by selected bacterial isolates in culture media supplemented with tryptophan (+Trp) and without tryptophan (-Trp). The values are the mean of three replicates and bars represent standard deviation. The columns bearing different letters are significantly different at $p < 0.05$ as calculated by Tukey's least significant difference (LSD) test.

3.1.8 Effect of pH and Temperature on Selected Bacterial Strains

The optimum pH for the growth of *B. megaterium* and *P. putida* was observed to be 7.0 while it was 8.0 for *B. tequilensis*. These studies also revealed that the higher pH restricted the growth of bacteria. Optimum temperature for the growth of the bacterial strains was found to be 37 °C while the extreme temperature (42 °C) restricted the growth of selected bacterial strains (Fig. 3.7).

3.1.9 Evaluation of PGPR on the Growth of Wheat Seedlings

In the current study, influence of PGPR on wheat seedlings was successfully evaluated. All three selected bacterial strains imposed a favorable impact on root length, shoot length and leaf area of wheat seedlings, under salinity stress. Bacterial strains significantly increased shoot and root length under saline conditions. Under stressful conditions, *B. tequilensis* stimulated root length and shoot length more than the control and other bacterial strains. The leaf area is directly proportional to the water status, growth and

photosynthetic activity of plant. When compared to the control, bacterial inoculation considerably improved the leaf area of wheat seedlings under stressful conditions. The inoculation impact of *B. tequilensis* was more obvious under induced salt stress condition than that of *B. megaterium* and *P. putida,* which could be attributed to strong production of ABA by *B. tequilensis,* that helps plants to mitigate negative impacts of salinity. The effect of bacterial inoculation on wheat seedlings demonstrated adequate nutritional uptake for nitrogen, phosphorus, and potassium. The uptake of N, P, and K was not efficient under control condition. Among all inoculated bacterial strains, the inoculation of *B. tequilensis* resulted in the maximum uptake of N, P, and K (Fig. 3.8, Appendix 2).

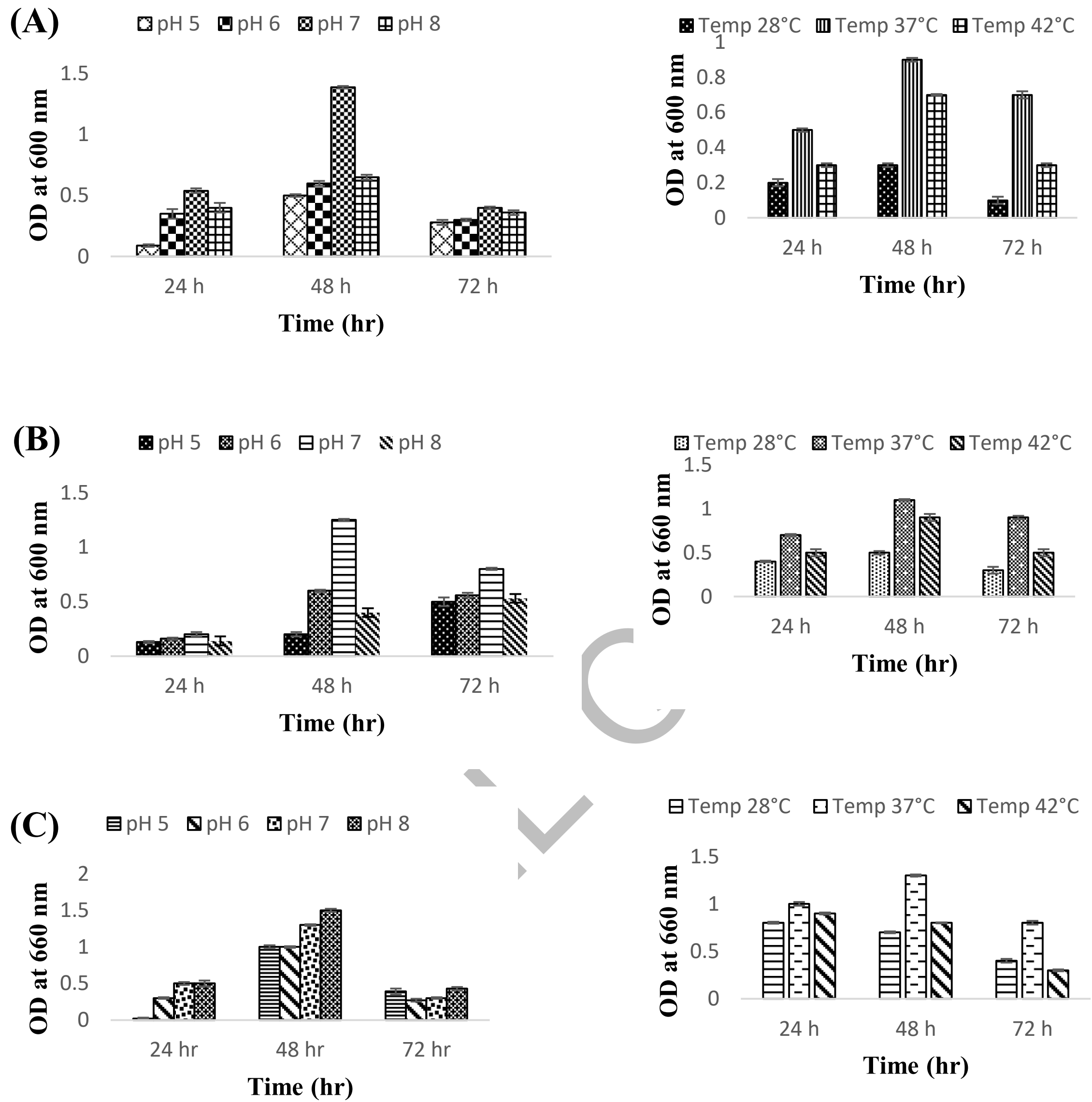

Fig. 3.7 Optimum pH and temperature conditions for the growth of three selected bacterial strains including *B. megaterium* (A), *B. tequilensis* (B) and *P. putida* (C). The values are the mean of three replicates and bars represent standard deviation.

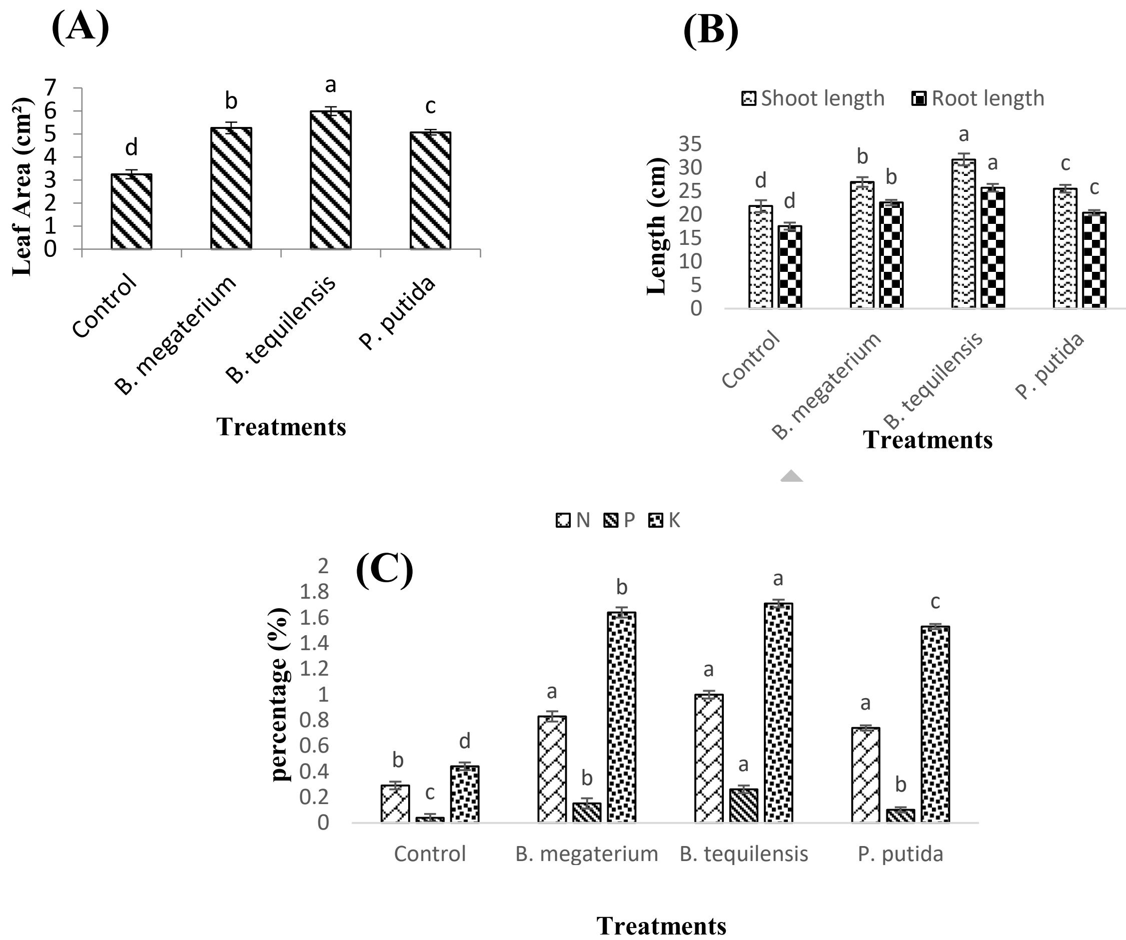

Fig. 3.8 Effect of PGPR on leaf area (A), root and shoot length (B), and nutrient uptake (C). The values are the mean of three replicates and bars represent standard deviation. The columns bearing different letters are significantly different at $p < 0.05$ as calculated by Tukey's least significant difference (LSD) test.

3.2 EXPERIMENT 2

3.2.1 Characterization of Plant Beneficial Traits

All tested bacterial strains were able to produce ACC deaminase, EPS and IAA and were also able to solubilize inorganic phosphate, even under salt stress condition (Fig. 3.9). The production of ACCD by the selected bacterial strains was ranging from 0.52 to 1.83 µM/mg protein/h. Among all tested strains, *B. tequilensis* was found to be more proficient and showed highest ACCD activity at 0 mM (1.83 µM/mg protein/h) and 25 mM salt concentration (1.70 µM/mg protein/h). In comparison, at 100 mM NaCl concentration, the strain synthesized 0.95 µM/mg protein/h. The range of IAA production by all three selected PGPR was 89.44–79.4 µM/ml. Moreover, *B. tequilensis* synthesized maximum amount of IAA in tryptophan supplemented media, under salt stress. The maximum PSI of *B. tequilensis* MPP8 was observed at 0 mM salt concentration (5.49) followed by 25 mM concentration (5.1) and 100 mM concentration (3.95). Under varying salt concentrations, strain *B. tequilensis* MPP8 was found to be more efficient in accumulating EPS than *B. megaterium* and *P. putida*. Maximum increase in EPS accumulation (1.33 mg/ml) was observed at 100 mM concentration by *B. tequilensis.*

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3.2.2 Salt Tolerance Assay of Selected Bacterial Strains, In Vitro

The growth rate of *B. megaterium* (MPP7), *B. tequilensis* (MPP8) and *P. putida* (MPP18) was successfully observed under different NaCl concentrations (0 mM, 25 mM, 50 mM and 100 mM) for seven days (Fig. 3.10). All the selected bacterial strains exhibited variable potential of salt tolerance. Till 5th day of incubation, both species of *Bacillus* revealed highest growth rate and it declined, thereafter. *P. putida* showed highest growth till 4th day of incubation, under different concentrations of NaCl.

3.2.3 Germination Percentage

Bacterial strains were successfully inoculated with seed priming and primed seeds were germinated in soil. The texture of the soil was loamy with 6.3 pH and 0.005 ds/m EC. These characteristics declared this soil to be ideal for plant growth. In the bacterial inoculated treatments, the germination rate of seeds was observed to be more than control. Increasing concentration of salt negatively affected the seed germination. The minimum

germination rate was observed in control treatment at 100 mM concentration of salt. Increase in germination rate was observed in bacterial inoculated treatments. The germination rate was in the subsequent order B2 > B1 > B3 > C and the maximum germination percentage was observed in B2 treatment, even at 100 mM salt concentration (Fig. 3.11).

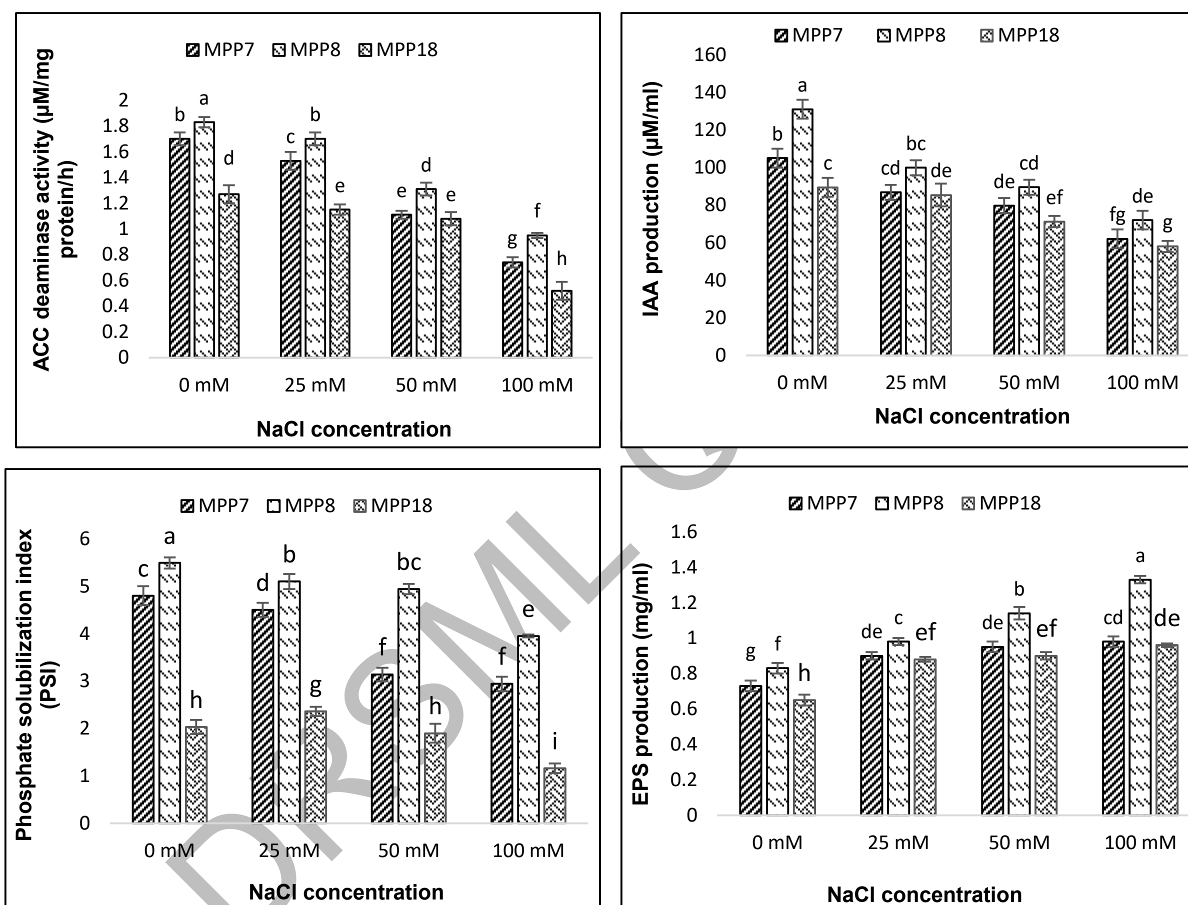

Fig. 3.9 Analysis of plant growth promoting properties of *B. megaterium* (MPP7), *B. tequilensis* (MPP8) and *P. putida* (MPP18). Under different salt stress conditions, ACC deaminase activity (a), IAA production (b), Phosphate solubilization index (c) EPS production (d) were observed. Values are described as means and bars denote standard errors. Dissimilar alphabets demonstrate significantly different values (P<0.05) from each other, as calculated by Tukey's least significant difference (LSD) test.

3.2.4 Biochemical and Physiological Parameters of Plants

Effect of treatments on growth of wheat seedling are presented in Appendix 3. Different biochemical and physiological parameters helped us to understand the response of plants under various treatments. Significant elevations of proline and sugar contents were observed with increasing concentrations of NaCl, in all bacterial inoculated treatments (B1, B2 and B3). Among these, B2 treatment resulted in maximum increase of both proline and sugar contents (Fig. 3.12A).

Salinity stress directly affected the growth of wheat seedlings. Among all treatments, the minimum root length and shoot length were observed in control (C treatment) at 100 mM NaCl concentration. The maximum root length and shoot length was observed in B2 treatment, under variable concentrations of salt (Fig. 3.12B). It was obvious from the growth analyses that the inoculation of bacterial strains significantly increased the fresh and dry weight of plants in both stressed and non-stressed conditions (Fig. 3.12C and D).

The application of bacterial strains triggered the production of photosynthetic pigments, and these were decreased under salinity stress condition. Similar effects of salt stress on carotenoid content were also observed (Fig. 3.12E).

RWC of wheat plants was reduced significantly with the increasing concentration of NaCl. By the application of PGPR, noticeable increase in RWC under salt stress condition was observed. The maximum RWC was observed in treatment B2, even under higher salt stress conditions. Salinity stress significantly increased electrolytic leakage. Priming of seeds with bacterial strains resulted in significant reduction of electrolytic leakage in all treatments (Fig. 3.12F). The salt tolerance index of wheat plants was significantly reduced under salinity conditions. However, PGPR inoculation increased salt tolerance index and the maximum salt tolerance was observed in *B. tequilensis* treated plants (Fig. 3.12G).

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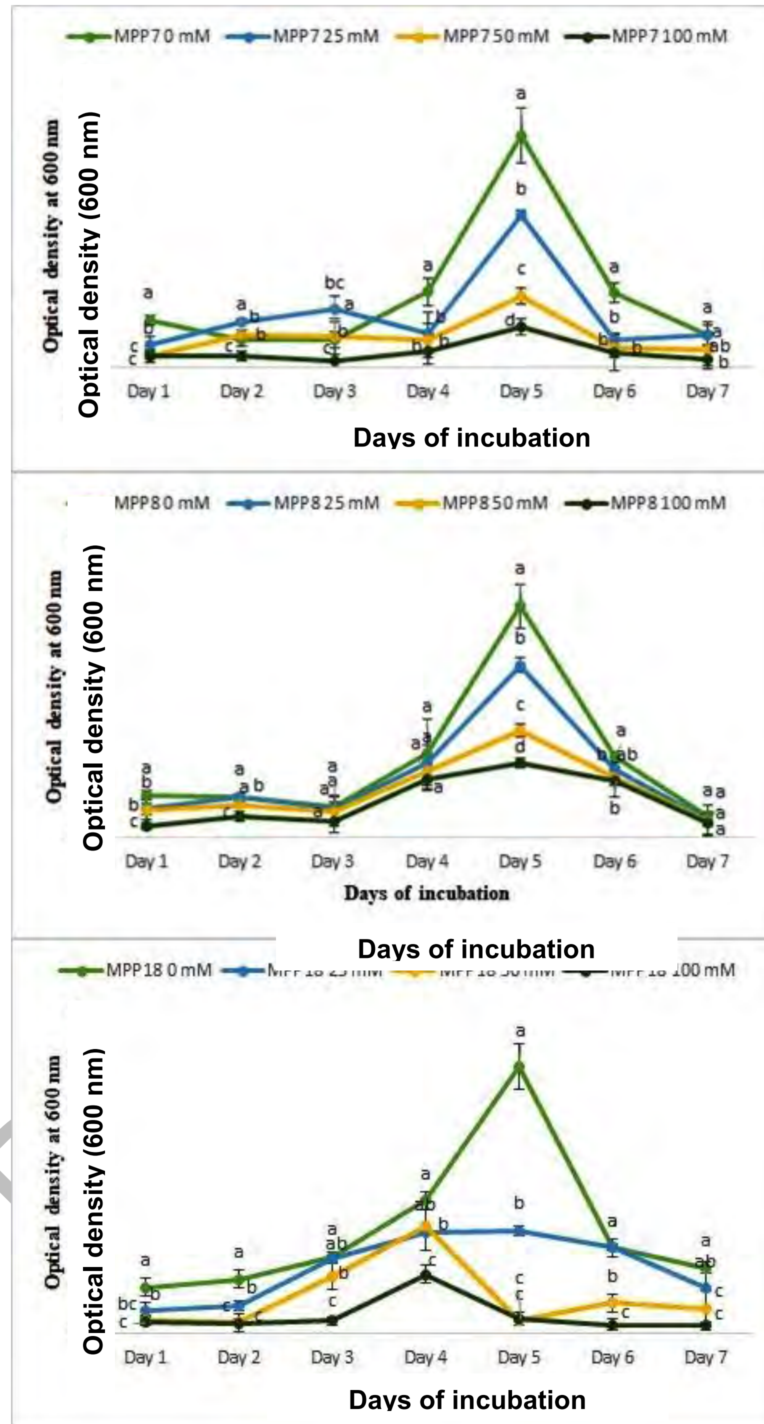


Fig. 3.10 Growth curve of *B. megaterium* (MPP7), *B. tequilensis* (MPP8) and *P. putida* (MPP18) under different salinity stress levels (0 mM, 25 mM, 50 mM and 100 mM). Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values ($P<0.05$) from each other, as calculated by Tukey's least significant difference (LSD) test.

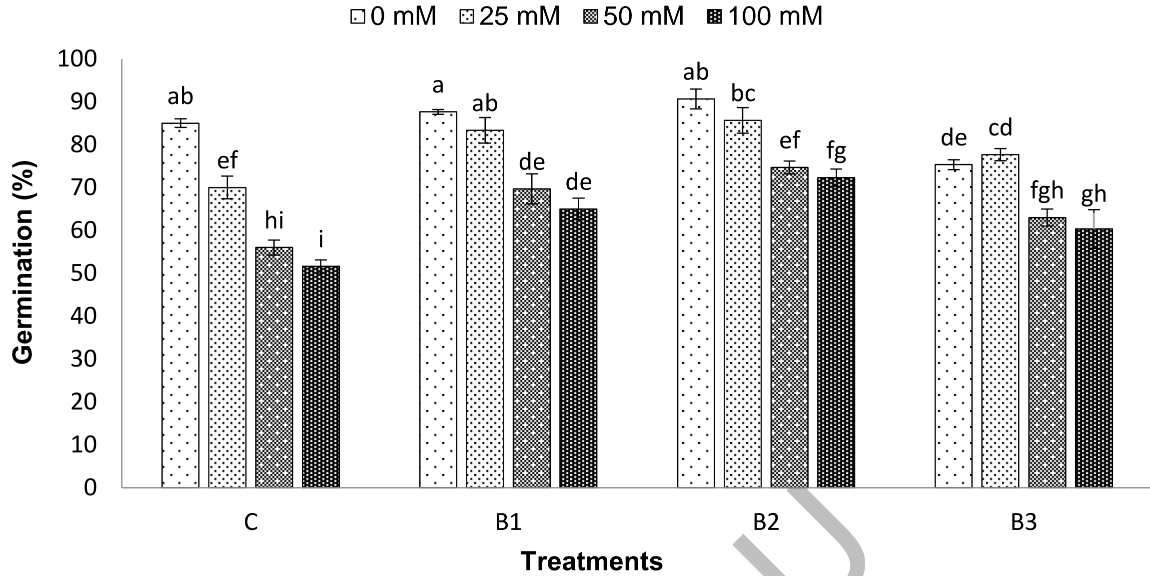
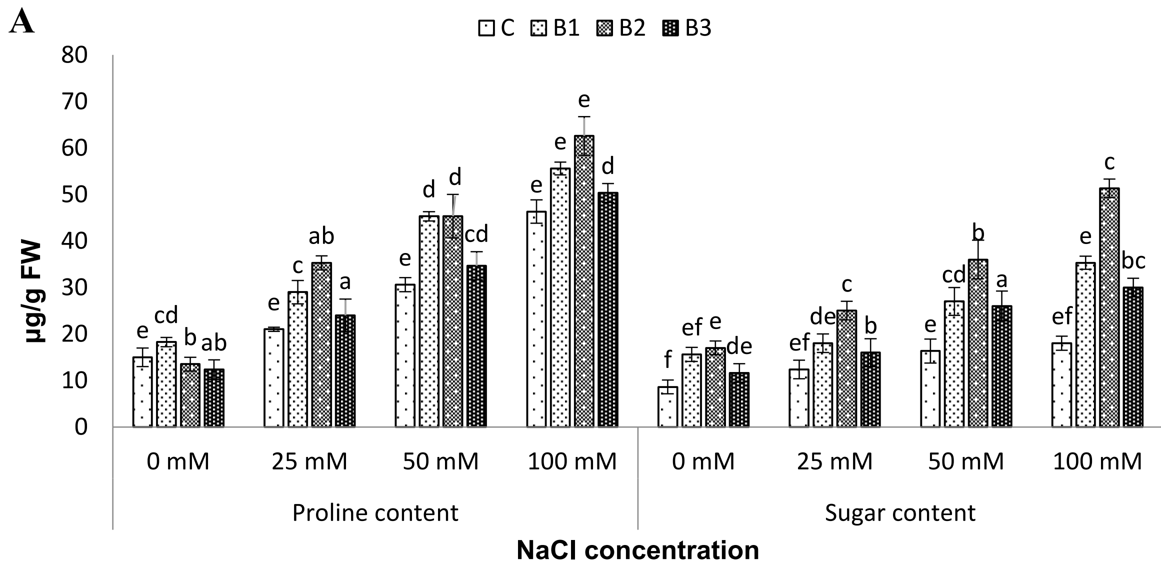
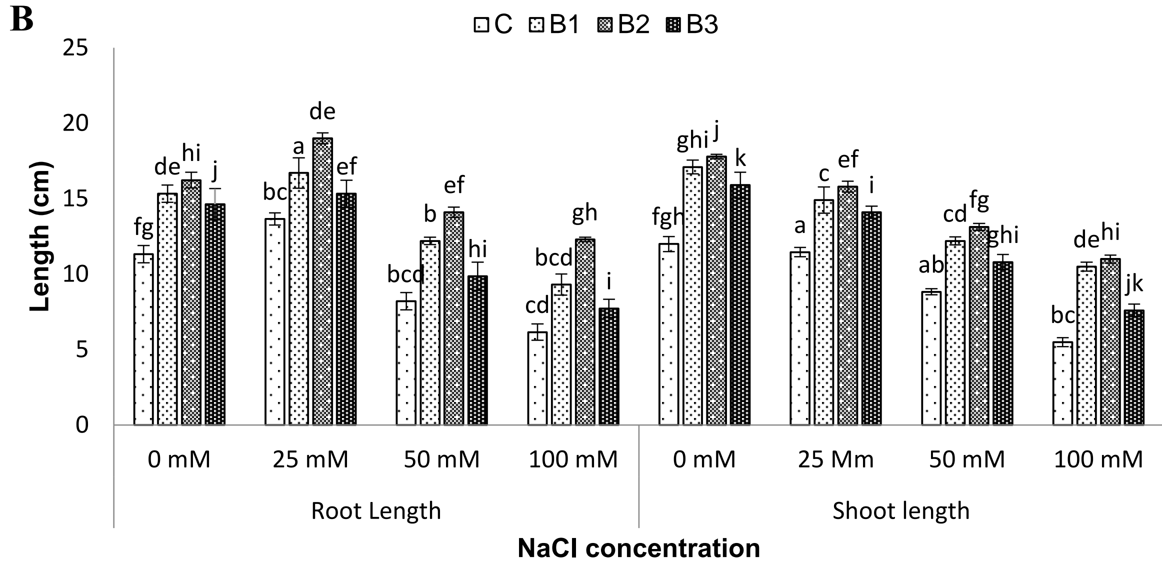


Fig. 3.11 Influence of various treatments on seed germination. Four different treatments including soil containing non-primed (normal) seeds (C), soil containing seeds primed with *B. megaterium* (B_1), soil containing seeds primed with *B. tequilensis* (B_2) and soil containing seeds primed with *P. putida* (B_3) were used under various concentrations (0-100 mM) of NaCl. Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values (P<0.05) from each other, as calculated by Tukey's least significant difference (LSD) test.




B
C B1 B2 B3
Length (cm)
25
20
15
10
5
0
fg de hi j
bc a de ef
bcd b ef hi
cd bcd gh i
fgh ghi j k
a c ef i
ab cd fg ghi
bc de hi jk
0 mM
25 mM
50 mM
100 mM
0 mM
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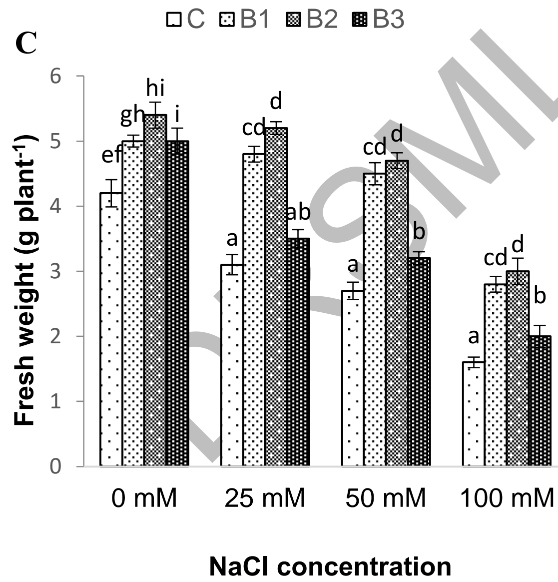
50 mM
100 mM
Root Length
Shoot length
NaCl concentration


C
C B1 B2 B3
Fresh weight (g plant-1)
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5
4
3
2
1
0
ef gh hi i
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a cd d b
0 mM
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50 mM
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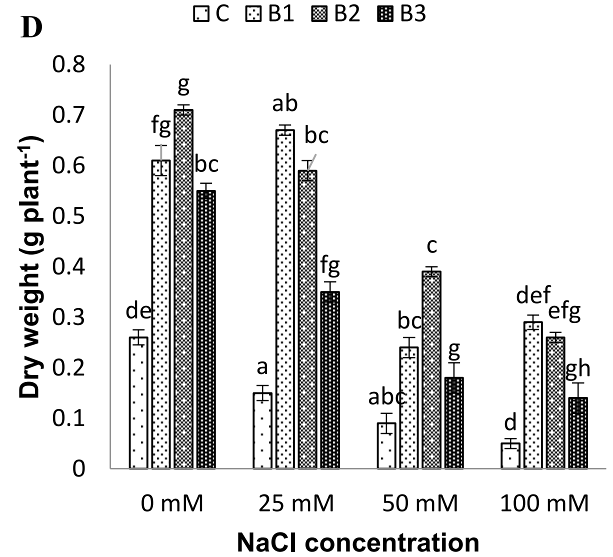
NaCl concentration


D
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Dry weight (g plant-1)
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0.7
0.6
0.5
0.4
0.3
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50 mM
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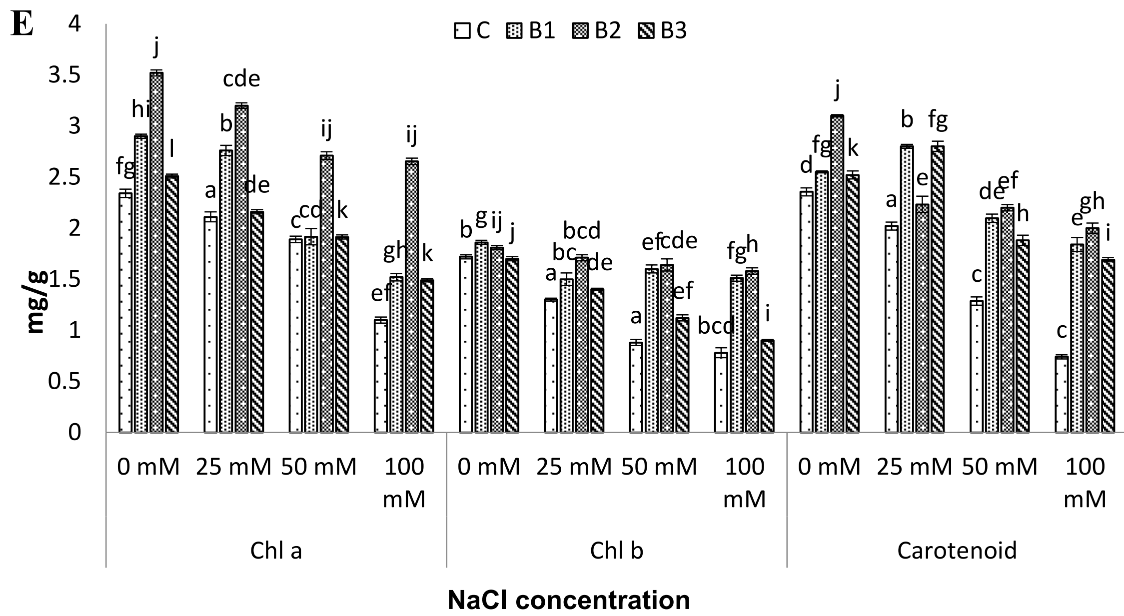
NaCl concentration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mg/g
NaCl concentration
0 mM
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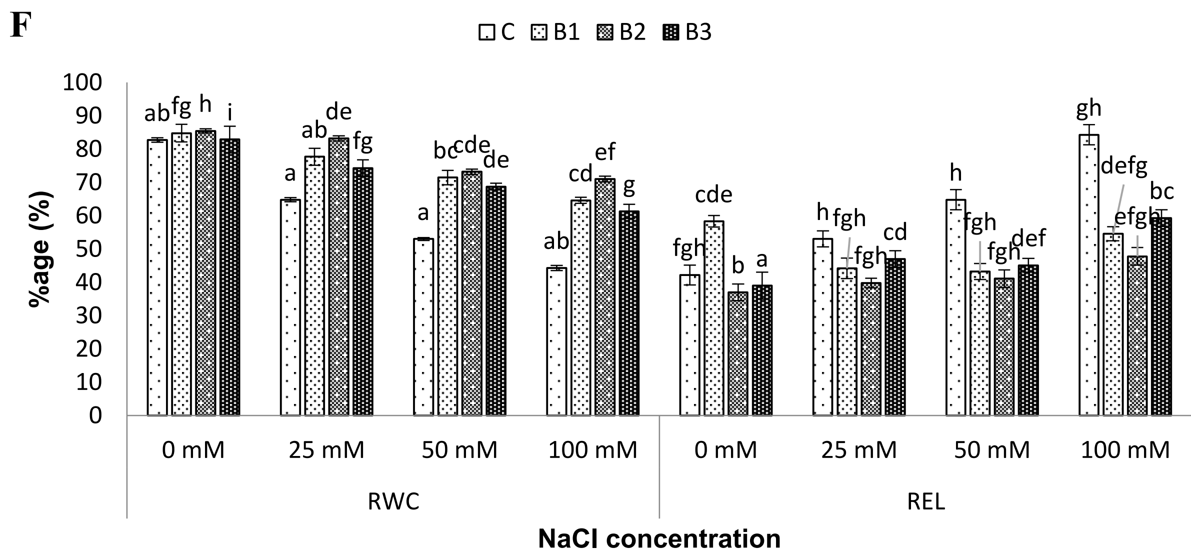
50 mM
100 mM
Chl a
Chl b
Carotenoid


F
C B1 B2 B3
%age (%)
NaCl concentration
0 mM
25 mM
50 mM
100 mM
RWC
REL

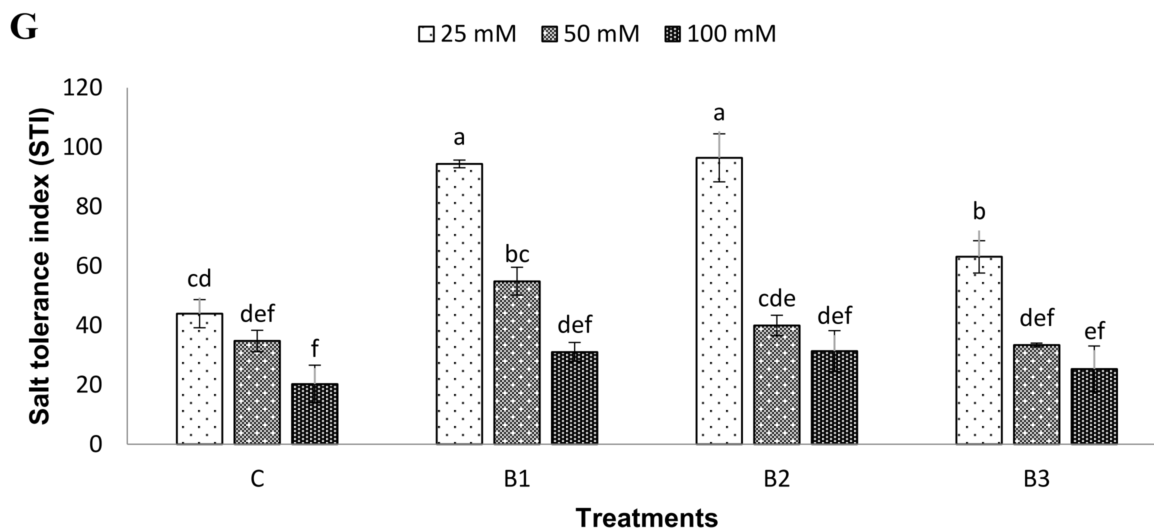


Fig. 3.12 Effects of different treatments on various biochemical and physiological parameters of PGPR inoculated wheat seedlings under various levels of salinity stress (0 mM, 25 mM, 50 mM and 100 mM). Four treatment including soil containing non-primed (normal) seeds (C), soil containing seeds primed with *B. megaterium* (B1), soil containing seeds primed with *B. tequilensis* (B2) and soil containing seeds primed with *P. putida* (B3) were used. Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values ($P<0.05$) from each other, as calculated by Tukey's least significant difference (LSD) test. RWC: Relative water content, REL: Relative electrolyte leakage.

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3.2.5 Antioxidant Enzymes Activity

The production of antioxidant enzymes was significantly increased in PGPR inoculated plants, under salt stress conditions (Fig. 3.13). Among all bacterial strains, the highest enzymatic activities were exhibited by *B. tequilensis* at 100 mM salt stress.

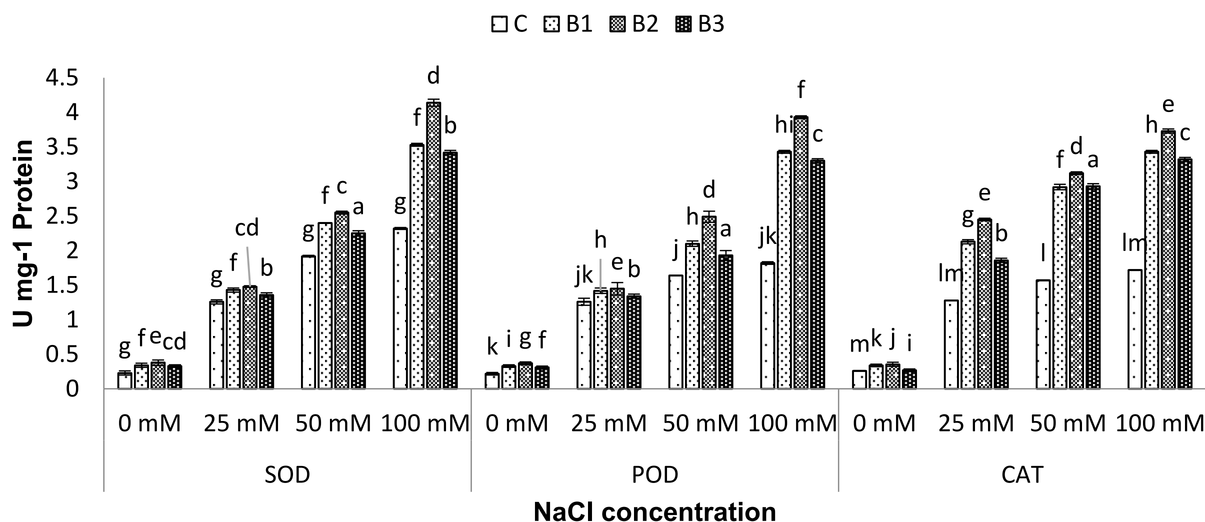

Fig. 3.13 Antioxidant enzymatic activities in PGPR inoculated wheat seedlings under various levels of salinity stress (0 mM, 25 mM, 50 mM and 100 mM). Four treatment including soil containing non-primed (normal) seeds (C), soil containing seeds primed with *B. megaterium* (B1), soil containing seeds primed with *B. tequilensis* (B2) and soil containing seeds primed with *P. putida* (B3) were used. Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values (P<0.05) from each other, as calculated by Tukey's least significant difference (LSD) test.

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3.2.6 Oxidative Burst

In the present study, under 100 mM salt stress condition, increased production of MDA and H_2O_2 was observed in wheat seedlings. Though, the inoculation of all PGPR decreased their production, *B. tequilensis* (B2) was more useful in reducing the levels of MDA and H_2O_2 (Fig. 3.14).

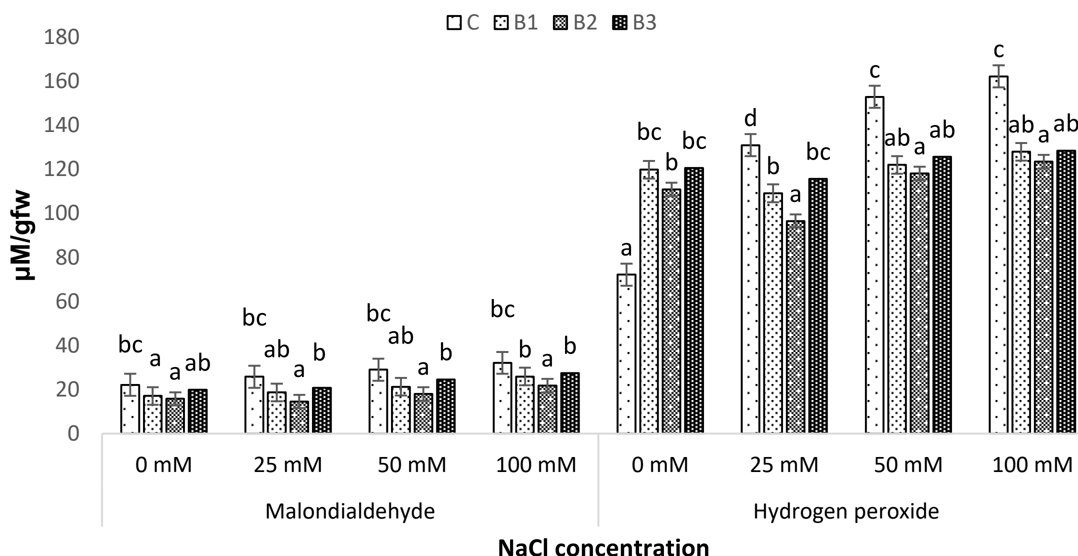

Fig. 3.14 Changes in malondialdehyde and hydrogen peroxide production in PGPR inoculated wheat seedlings under various levels of salinity stress (0 mM, 25 mM, 50 mM and 100 mM). Four treatment including soil containing non-primed (normal) seeds (C), soil containing seeds primed with *B. megaterium* (B1), soil containing seeds primed with *B. tequilensis* (B2) and soil containing seeds primed with *P. putida* (B3) were used. Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values ($P<0.05$) from each other, as calculated by Tukey's least significant difference (LSD) test.

3.2.7 Expression Analysis of Stress Related Genes

For better understanding of plant defense mechanism, the expression levels of two stress-related genes viz. SOS1 and SOS4 were observed in wheat plants under salinity and PGPR treatments. In this trial, only the most efficient strain (*B. tequilensis* MPP8) was used. Application of PGPR significantly affected the relative expression of SOS1 and SOS4 genes, in the leaves of wheat seedlings (Fig. 3.15). A higher expression of these genes was observed after salt stress and bacterial inoculation. As compared to control, the application of *B. tequilensis* enhanced the expression of SOS1 and SOS4 protein genes and depicted their potential role in salinity tolerance.

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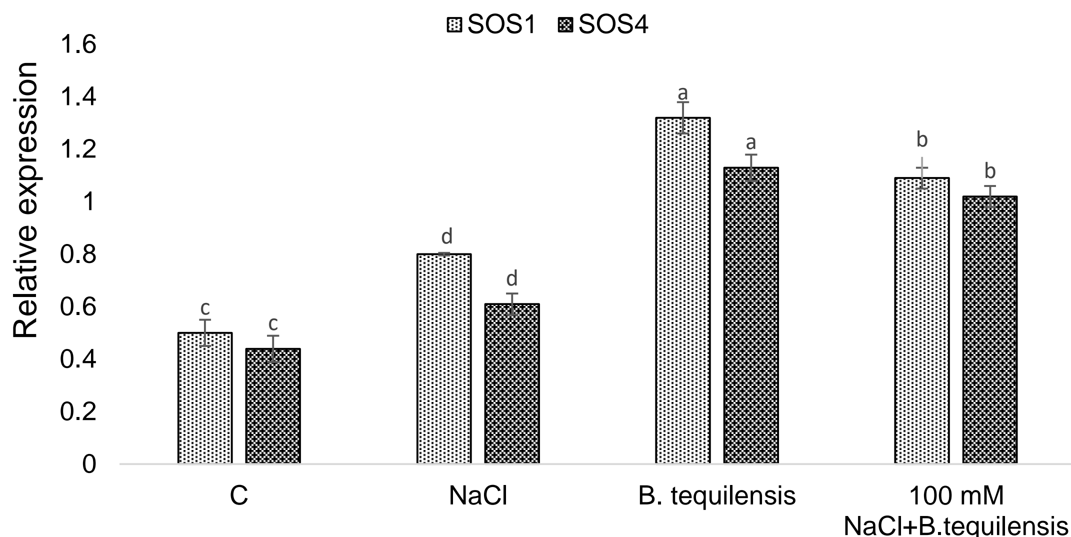

Fig. 3.15 Relative expression of SOS1 and SOS4 genes, in the leaves of wheat plants. Relative expression was observed in plants grown without PGPR and salt stress (C), plants grown under salt stress (NaCl), plants inoculated with halotolerant PGPR (*B. tequilensis*) and plants grown under salinity stress and inoculated with *B. tequilensis* (100 mM NaCl + *B. tequilensis*). Bars denote standard errors. Dissimilar alphabets demonstrate significantly different values (P<0.05) from each other, as calculated by Tukey's least significant difference (LSD) test.

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3.2.8 Principal Component Analysis (PCA)

PCA confirmed the role of PGPR in the growth of wheat plant under different stress conditions (Fig. 3.16). The biplot among the charted statistics (F1 and F2) showed 83.66% differences (F1 presented about 61.58% and F2 presented about 22.08% differences). Blue color dots are indicating correlation among the different experimental treatments and red dots are representing the correlation among different variables. Positively correlated variables were found to be located near to each other in the same quadrant. Bioplot revealed that plant growth attributes, photosynthetic pigments, RWC and STI are positively correlated with each other and negatively correlated with all other parameters.

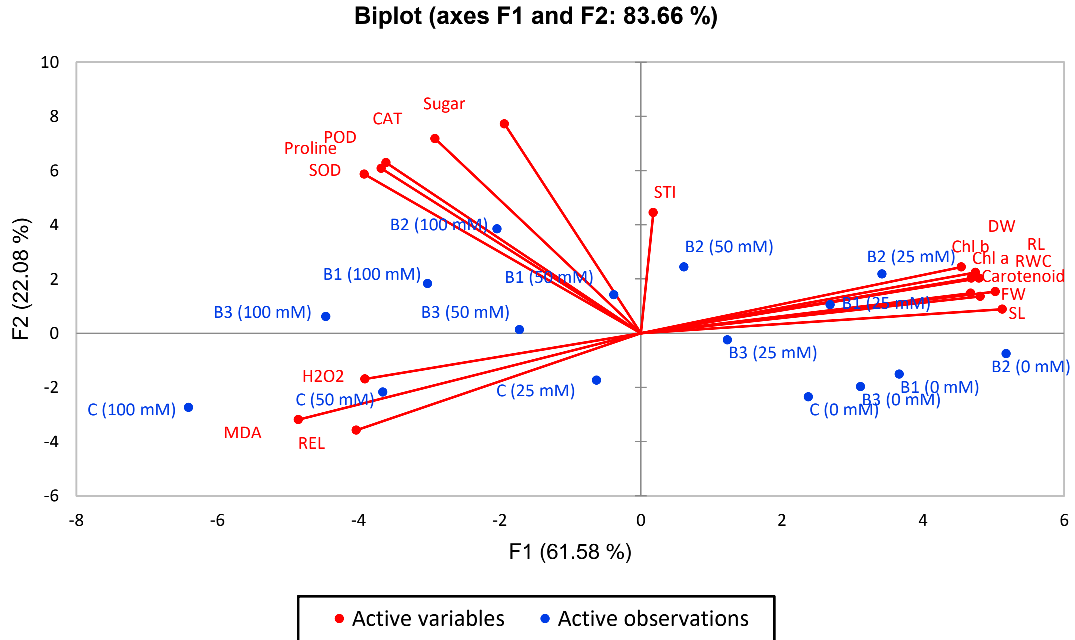

Fig. 3.16 Pearson correlation biplot among the charted statistics (F1 and F2). Blue color dots are indicating correlation among the different experimental treatments and red dots are representing the correlation among different variables.

3.3 EXPERIMENT 3

3.3.1 Bacterial Flocculation

Bacterial flocculation is the aggregation of dispersed bacterial cells into flocs or flakes. Floc yield at varying concentrations of NaCl was recorded in mg/L. It was observed that floc yield increased with increasing concentration of NaCl, and maximum floc yield was observed at 100 mM concentration of NaCl (Fig. 3.17A).

3.3.2 Biofilm Formation

B. tequilensis was found to be capable of producing biofilm under varying salinity levels. Biofilm formation increased at increasing salinity levels and the greatest biofilm was formed at 100 mM concentration of NaCl (Fig. 3.17B).

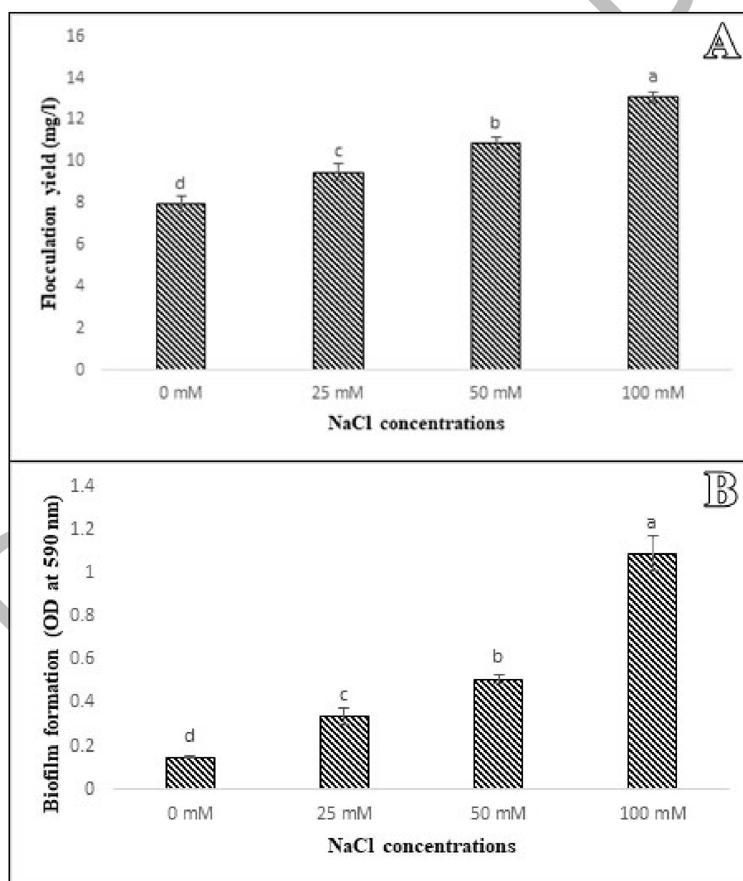

Fig. 3.17 The effect of varying levels of salt on the production of bacterial flocs (A) and biofilm formation (B). Standard errors are represented by bars. Means with different letters varied significantly from each other at $p<0.05$.

3.3.3 Scanning Electron Microscopic Observation

The scanning electron microscopic photographs under saline and control conditions were compared. *B. tequilensis* produced EPS under saline conditions. Under control conditions, cells were scattered, while these were formed and aggregated under NaCl stress, by the production of EPS (Fig. 3.18).

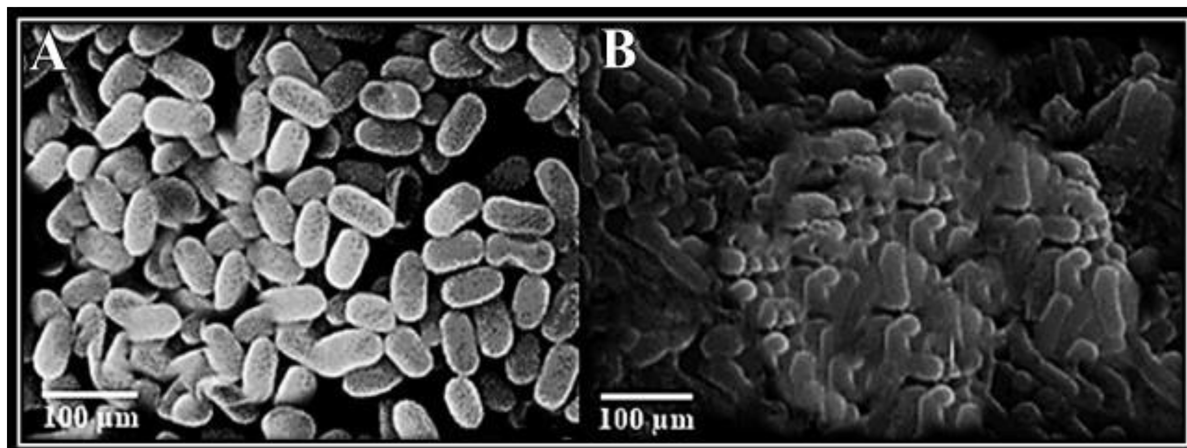

Fig. 3.18 SEM images of *B. tequilensis* cells under controlled (A) and saline (B) conditions.

3.3.4 Fourier Transform Infrared Spectroscopic (FTIR) Analysis

The main functional groups were successfully identified by FTIR spectroscopy. FTIR spectrum of *B. tequilensis*-EPS revealed characteristic absorption peaks of polysaccharides. Peak at 3245 cm^{-1} indicated the presence of hydroxyl groups. As each monosaccharide has more than one hydroxyl group, it confirmed the presence of polysaccharides. The band at around 1400 cm^{-1} showed C-H stretching and angular vibration, indicating the presence of carbohydrates. C-N (aliphatic amines), C-Br (alkyl halide), and C=O stretching of carboxylate and amide groups (amide I band) were represented by the peaks at 664 cm^{-1}, 1085 cm^{-1}, and 1630 cm^{-1}, respectively. The flocculating activity could be attributed to the presence of hydroxyl, carboxylate and amino functional groups (Fig. 3.19).

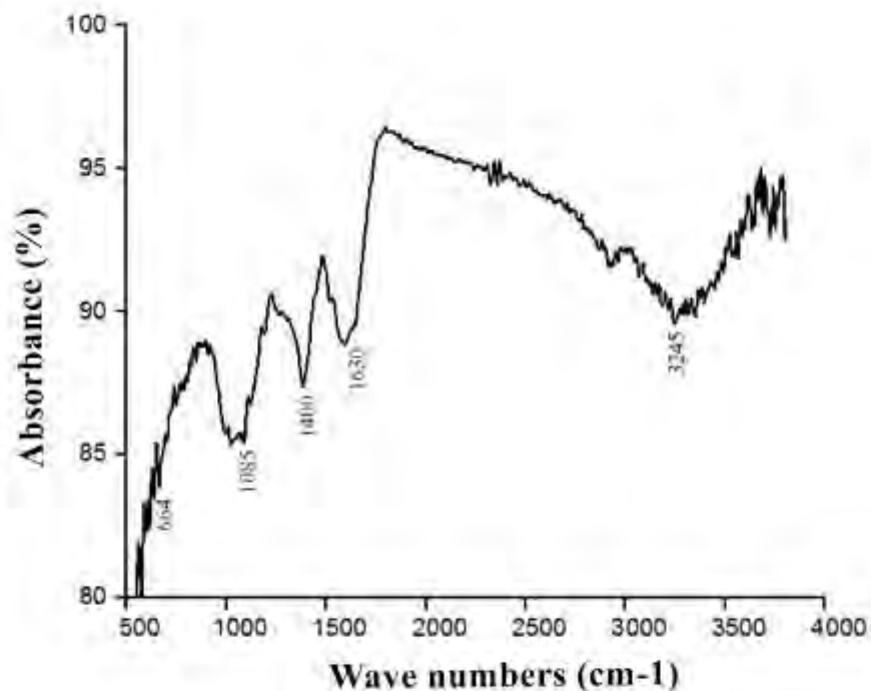

Fig. 3.19 Fourier transform infrared (FTIR) spectrum of *B. tequilensis*-EPS in 400–4000 cm^{-1} range, revealing common bands that are characteristic of polysaccharides.

3.3.5 Screening of Genes Conferring PGP Traits

PCR results revealed the presence of acdS and pqqE genes in the genome of *B. tequilensis* (Fig. 3.20). Presence of these genes indicated ACC deaminase and phosphate solubilizing ability of *B. tequilensis.*

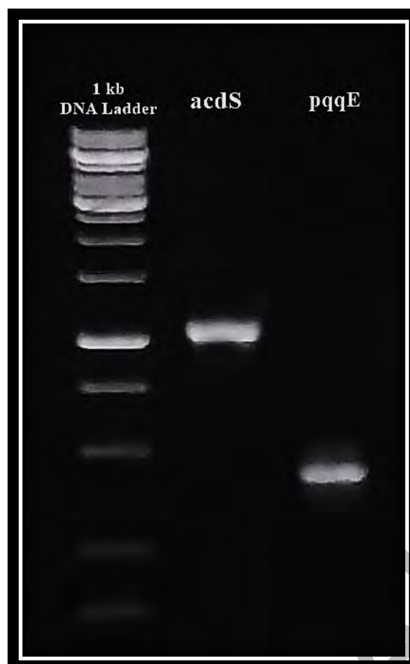

Fig. 3.20 Gel electrophoresis of amplified acdS and pqqE genes.

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3.3.6 Germination Rate

Effect of treatments on growth of wheat seedling are presented in Appendix 4. Increasing concentration of NaCl reduced germination rate of chickpea seeds. Treatment of *B. tequilensis* displayed positive effect and improved germination percentage of chickpea seeds (Fig. 3.21A).

3.3.7 Plant Analysis

Saline conditions negatively affected the lengths of shoots and roots of chickpea seedlings. Application of *B. tequilensis* reduced this effect, significantly. Positive influence of bacterial inoculation was obvious at all NaCl concentrations (Figs. 3.21B and C). Soil inoculation of *B. tequilensis* helped stressed plants to maintain high fresh weight of seedlings (Fig. 3.21D).

Salinity stress damaged the seedlings of chickpea and resulted in the higher leakage of electrolytes. Inoculation of *B. tequilensis* positively played its role to decrease this leakage (Fig. 3.21E). Salinity stress also decreased relative water content of leaves. Like other physiological parameters, the soil application of *B. tequilensis* helped chickpea seedlings to maintain higher RWC and grow better (Fig. 3.21F).

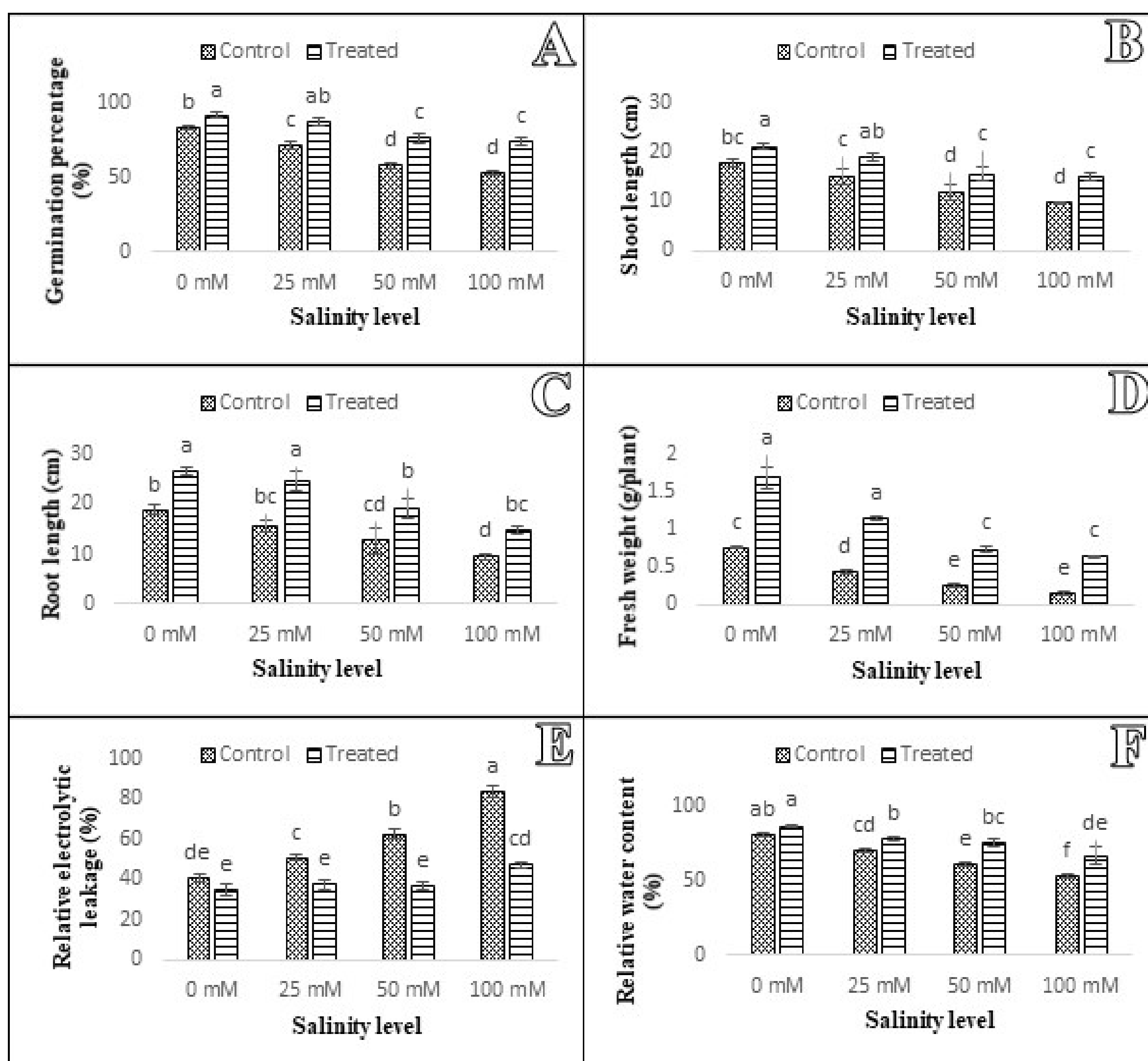

Fig. 3.21 Influence of different salt treatments on germination %age (A) shoot length (B), root length (C), fresh weight (D), relative electrolyte leakage (E) and relative water content (F) of chickpea seedlings. Standard errors are represented by bars. Means with different letters are significantly different from each other at $p<0.05$.

Inoculation of *B. tequilensis* also helped plants to maintain higher contents of chlorophyll and carotenoids (Fig. 3.22A and B). Deterioration of these pigments indicated plant damage. Their higher concentration described less damage of chickpea and elaborated the positive influence of *B. tequilensis.*

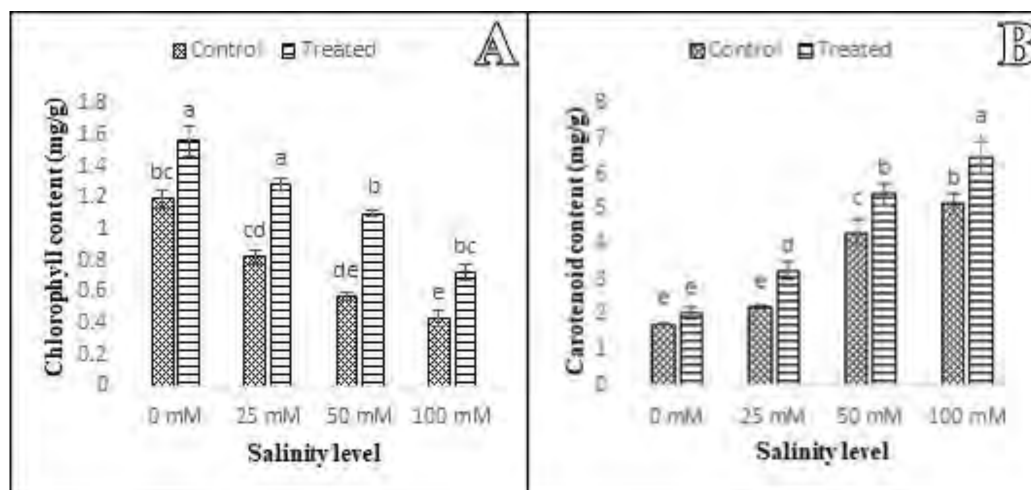

Fig. 3.22 Influence of different salt treatments on chlorophyll (A) and carotenoid contents (B) of chickpea seedlings. Standard errors are represented by bars. Means with different letters are significantly different from each other at $p<0.05$.

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Total soluble sugar and proline are osmolytes that serves as antioxidants. They are abundantly accumulated in plants under stressful conditions. Proline and TSS contents were estimated in control and treated plants subjected to varying levels of salinity stress. Both were observed to be increased under bacterial inoculated treatments, as compared to control. The plants treated with PGPR accumulated more osmolytes under saline conditions as compared to un-inoculated ones (Fig. 3.23).

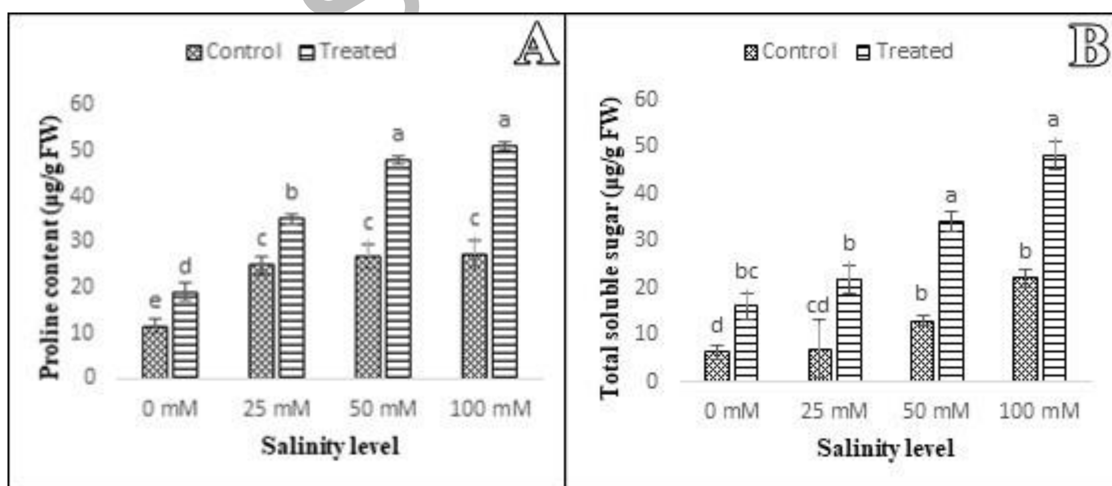

Fig. 3.23 Influence of different salt treatments on proline content (A) and total soluble sugars (B) of chickpea seedlings. Standard errors are represented by bars. Means with different letters are significantly different from each other at $p<0.05$.

Salt stress conditions triggered the production of all tested antioxidant enzymes (SOD, POD and CAT). Interestingly, the application of *B. tequilensis* further increased their concentrations at all salinity levels (Fig. 3.24). The greatest salinity-induced increase in SOD, POD, and CAT levels of chickpea seedlings was observed at 100 mM concentration of NaCl.

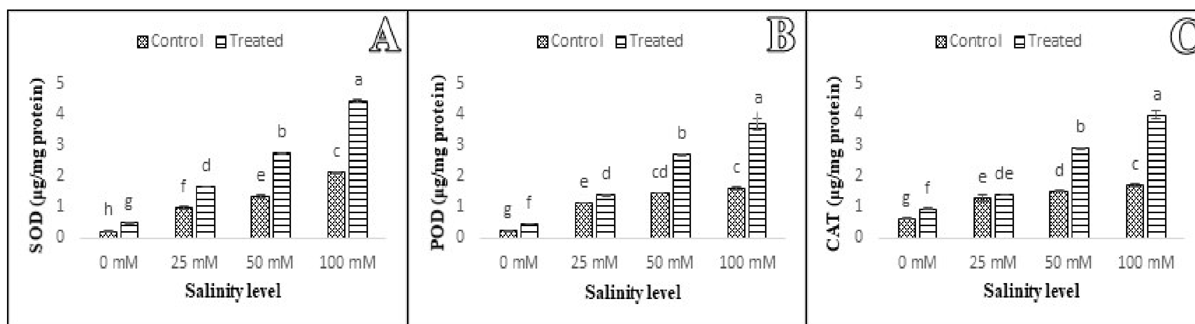

Fig. 3.24 Influence of different salt treatments on the production of important antioxidant enzymes including SOD (A), POD (B) and Catalase (C). Standard errors are represented by bars. Means with different letters are significantly different from each other at $p<0.05$.

Production of MDA and H_2O_2 was increased under salinity stress. Increasing salt concentrations resulted in elevated production of these harmful reactive compounds. Inoculation of soil with *B. tequilensis* resulted in the decreased accumulation of both MDA and H_2O_2 at all concentration of NaCl (Fig. 3.25).

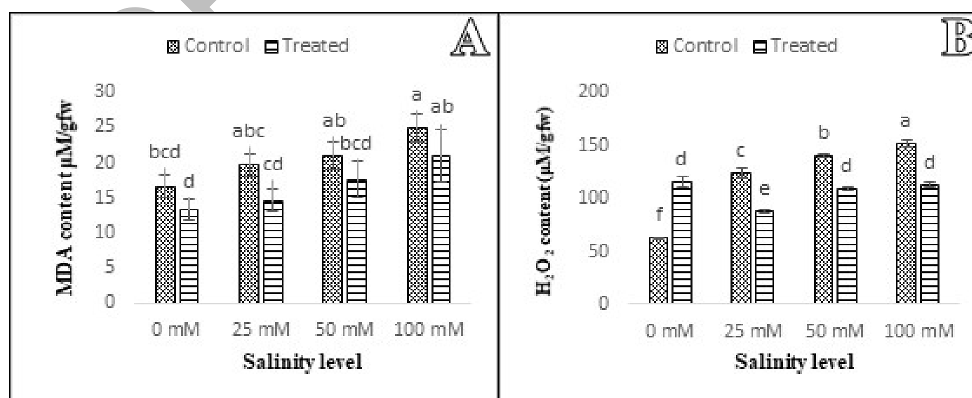

Fig. 3.25 Influence of different salt treatments on the production of MDA (A) and H_2O_2 (B) of chickpea seedlings. Standard errors are represented by bars. Means with different letters are significantly different from each other at $p<0.05$.

In comparison to the non-inoculated control, *B. tequilensis* inoculation significantly increased the salt tolerance indices of chickpea seedlings (Fig. 3.26).

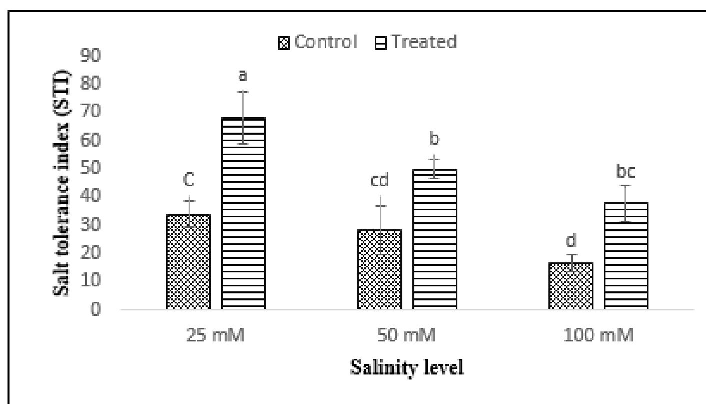

Fig. 3.26 Salt tolerance index of chickpea seedlings under control and treated conditions. Salt was provided in three different concentrations including 25 mM, 50 mM and 100 mM. Bars are representing standard errors. Means with different letters are significantly different from each other at $p<0.05$.

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3.3.8 Pearson Correlation Biplot

Positive influence of *B. tequilensis* inoculation on chickpea seedlings was confirmed by principal component analysis (PCA). Orange dots showed the correlation among different control and treated plants under normal and saline conditions. Whereas blue dots represented the correlation among different parameters. The variables that were clustered together in the same quadrant had positive correlation. Morphological parameters like chlorophyll content and RWC showed positive correlation with each other and had negative correlation with antioxidant enzymes, osmolytes, and oxidative compounds (Fig. 3.27).

3.3.9 Scatter Plot

Scatter plot depicted the correlation between parameters under 100 mM of NaCl stress, while correlation coefficients described the linkage of two variables. Correlation coefficient, larger than zero, indicated positive correlation, while negative correlation was signified by less than zero coefficient values. The zero-value signified no correlation. Chlorophyll contents and RWC had positive correlation among them, but they showed negative correlation with SOD, POD, CAT, H_2O_2 and MDA (Fig. 3.28).

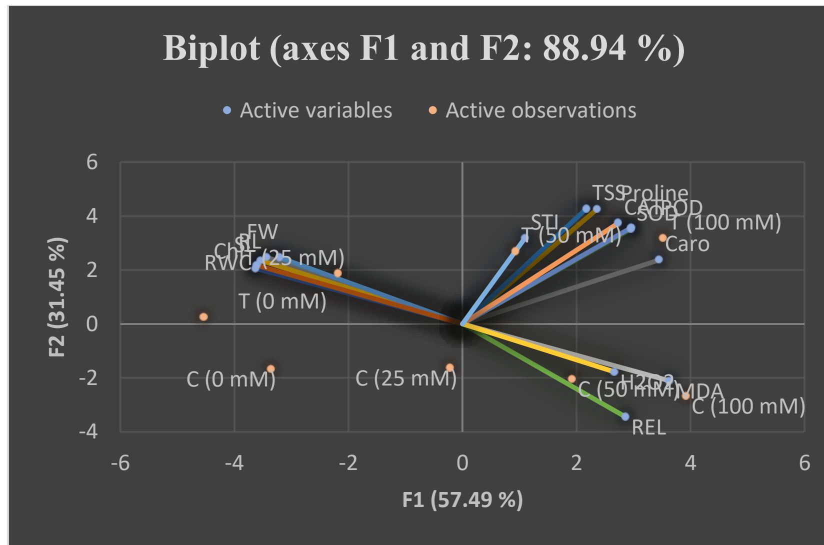

Fig. 3.27 Pearson correlation biplot amongst plotted statistics (F1 and F2). Oranges spots reflect correlation between various investigated treatments, while blue spots depict correlation among various studied parameters.

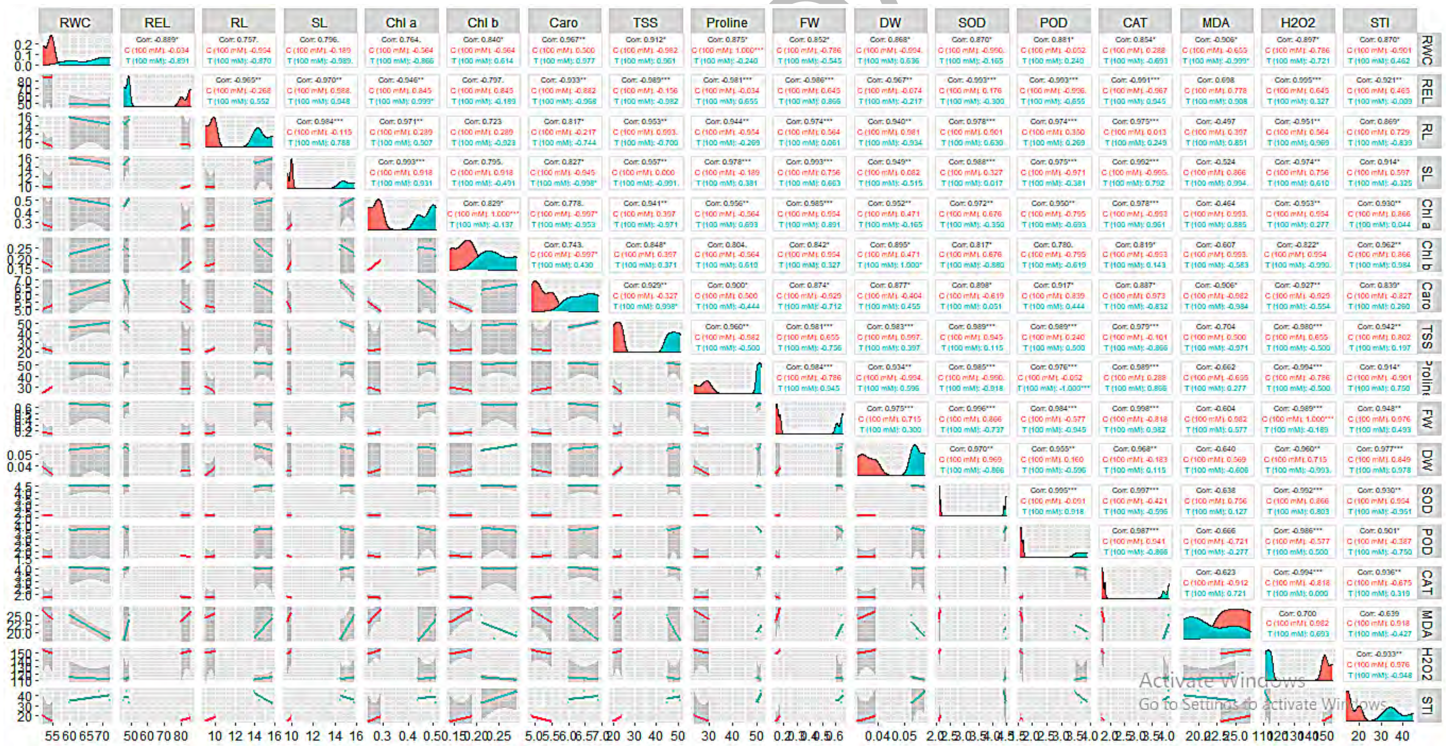

Fig. 3.28 Correlation analysis of studied parameters including RWC (relative water content), REL (relative electrolytic leakage), RL (root length), SL (shoot length), FW (fresh weight), Chl (total chlorophyll), STI (salt tolerance index), TSS (total soluble sugar), Caro (carotenoids), Proline, POD (peroxidase), SOD (superoxide dismutase), CAT (catalase), MDA (malondialdehyde) and H_2O_2 (hydrogen peroxide) at 100 mM of NaCl.

3.4 EXPERIMENT 4

3.4.1 Gas Exchange Measurements

For gas exchange metrics, there was a variation among bacterial treated seedlings under salinity stress conditions. When compared to control, four weeks of salt exposure at 100 mM NaCl caused highly significant alterations in these parameters. The statistical analysis of data related to gas exchange properties showed a significant impact of bacterial treatment at different salt stress levels (Fig. 3.29). Data on gas exchange properties revealed that salinity stress and bacterial inoculation influenced the response of chickpea. Salinity stress resulted in a considerable decrease in net CO_2 assimilation, intracellular CO_2 concentration, stomatal conductance, net photosynthetic rate, and transpiration rate as well as a rise in vapor pressure deficit and leaf temperature. Salinity stress damaged uninoculated plants significantly at 100 mM stress level, when compared to bacterial inoculation plants and untreated plants.

3.4.2 Stomatal Assay

In comparison to stress treatment, *B. tequilensis* colonization in saline conditions increased stomatal and pore length while decreasing pore and stomatal width. Stomata and pore width were greater in normal plants, whereas stomata and pore length were greater in salt stressed plants (Fig. 3.30). Under salt stress situation, closed stomata and distorted guard cells could also be observed (Fig. 3.31).

3.4.3 Leaf Injury Index

At the end of the experiment, injury symptoms were observed (Fig. 3.32). Qualitative observation revealed necrosis and abscission of salt stress on the leaves. When compared with control and bacterial inoculated treatment, it was observed that salt stressed plants were severely affected by necrosis and were confined to fewer leaves. Control plants were looking healthier and greener. The plants in treatment S+PGPR were looking healthier than S treatment. It depicts that bacterial inoculation has reduced the severity of salt stress (Fig. 3.33).

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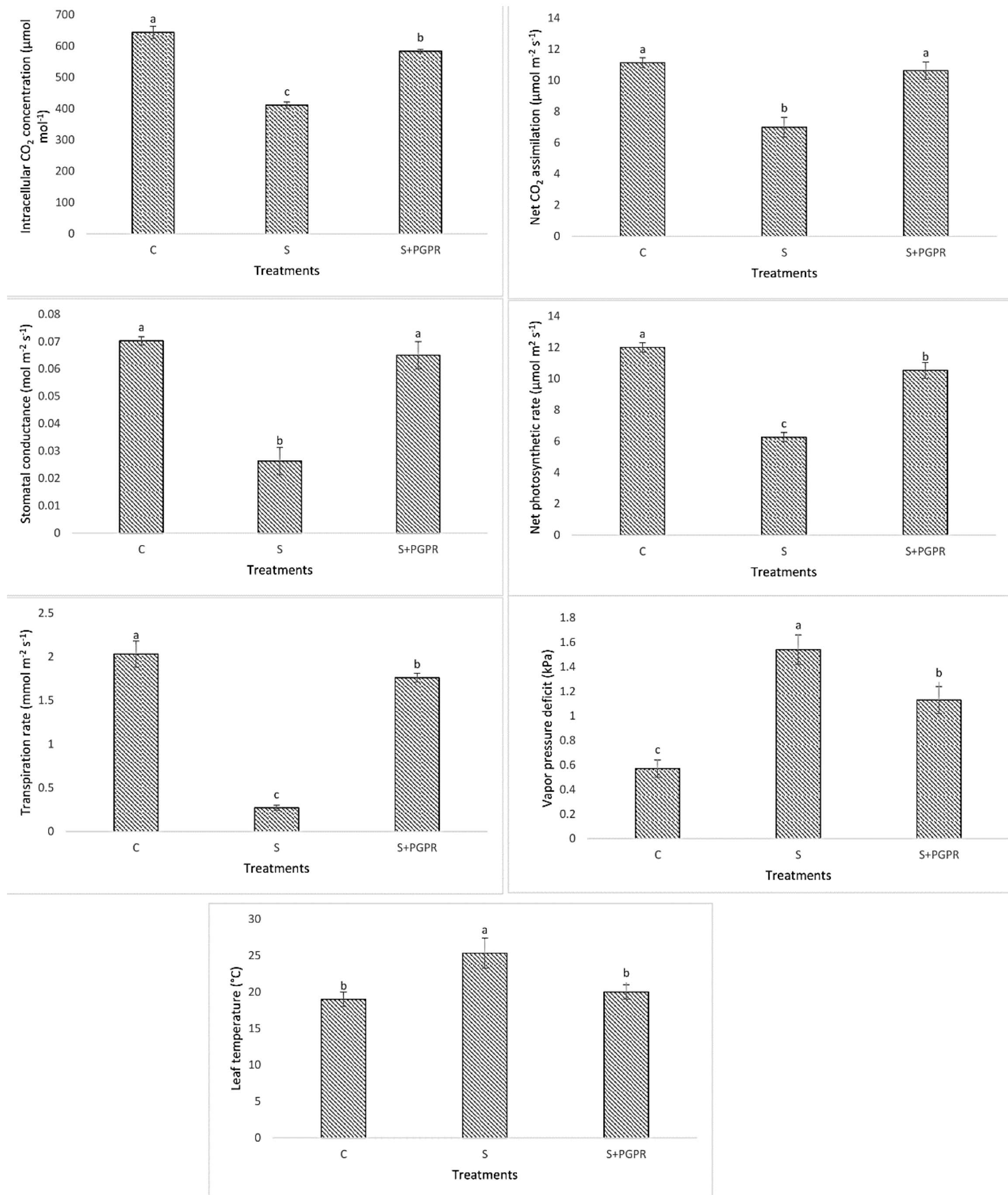

Fig. 3.29 In different treatments, parameters like intracellular CO_2 concentration (A), net CO_2 assimilation (B), stomatal conductance (C), net photosynthetic rate (D), transpiration rate (E), vapor pressure deficit (F) and leaf temperature (G) were observed for 4 weeks. Data are mean ± SE (n = 3). Different alphabets indicate significant difference between different treatments.

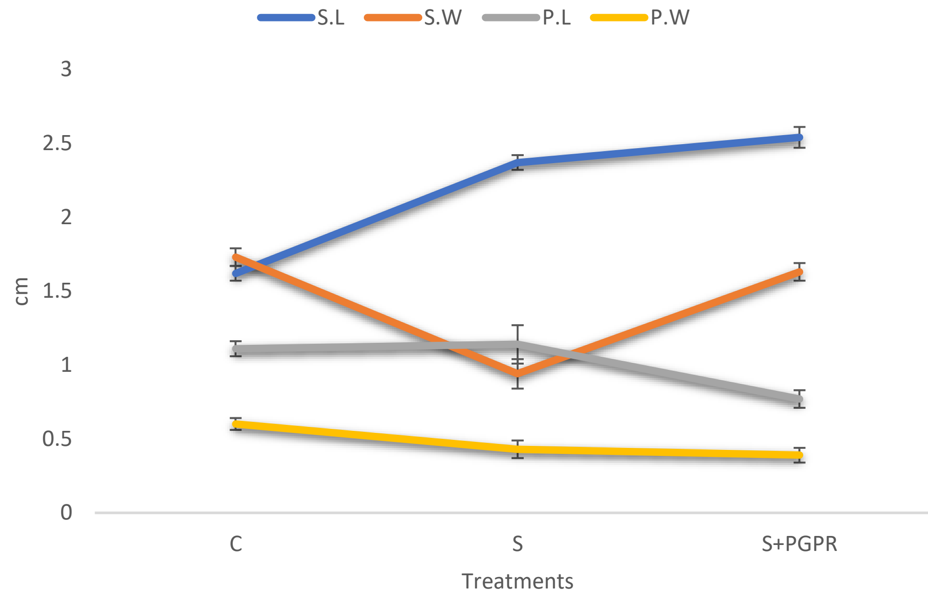

Fig. 3.30 Stomatal traits including stomatal length (SL), stomatal width (SW), pore length (PL) and pore width (PW) were measured. Bars represents the standard errors.

Fig. 3.31 Response of stomata in 40 days old *C. arietinum* leaves under various treatments. Control (A), 100 mM NaCl (B) and *B. tequilensis*+100 mM NaCl (C).

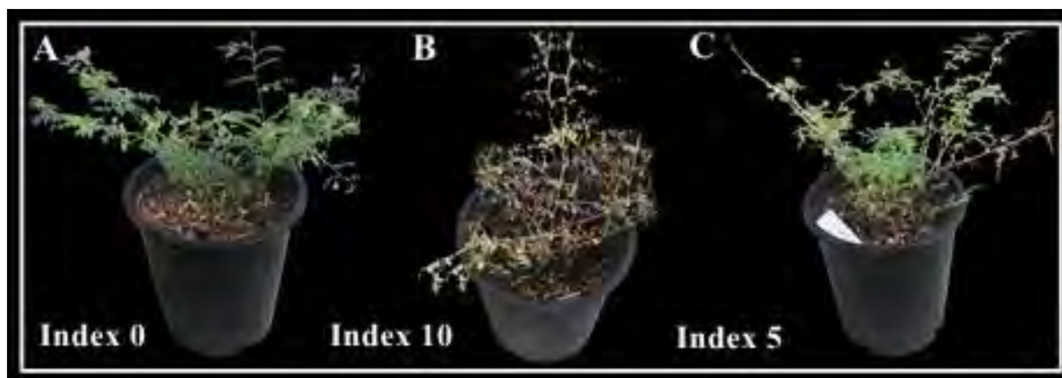

Fig. 3.32 The damage rating system was used to quantify visual assessments of saline symptoms in different treatments. Plants with no signs of salt harm received a score of 0; dead plants received a score of 10. Plants were given an extremely high concentration of saline solution (100 mM), till they died.

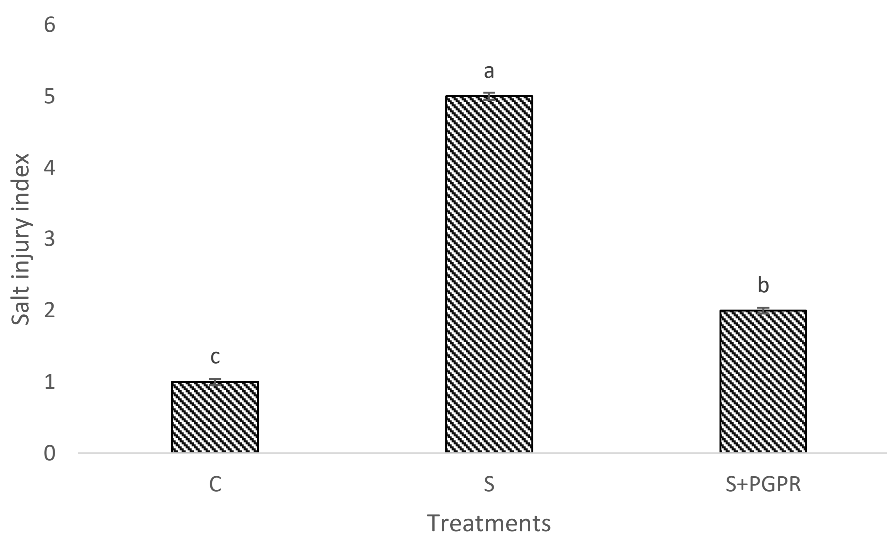

Fig. 3.33 Effects of NaCl concentration on salt injury index of *C. arietinum* seedlings.

3.4.4 Leaf Osmolality

The results showed that when leaves were exposed to salt stress, Na^+ uptake rose dramatically, at 100 mM salt stress, while K^+ uptake decreased, resulting in an elevated Na^+/K^+ ratio. In comparison to uninoculated plants under salt stress, Na^+ concentration declined by 22% and the K^+ absorption increased by 45% at 100 mM salt concentration. At the same time, PGPR treatment reduced Na^+/K^+ ratio at 100 mM salt treatment (Table 3.7).

Table 3.7: Effect of halotolerant *B. tequilensis* treatment on sodium and potassium content of chickpea grown under salinity stress.

Treatments	Na^+ (mg g^{-1}dw)	K^+ (mg g^{-1}dw)	Na^+/K^+ ratio
C	3.80 ± 0.76 b	14.9 ± 2.0 a	0.25 ± 0.09
S	37.6 ± 1.59 a	8.00 ± 1.9 b	4.7 ± 0.81
S+PGPR	18.9 ± 2.01 c	13.1 ± 1.5 a	2.2 ± 0.17

3.4.5 Correlation between Different Studied Parameters

As shown in Figure 5, salt injury index was negatively correlated with CO_2 assimilation, stomatal conductance, intracellular CO_2 concentration, transpiration net photosynththic rate, stomatal and pore width. However, its positive correlation was observed with vapor pressure deficit, leaf temperature, sodium potassium ratio, and stomatal and pore length. It was observed that no strong correlation exist between salt injury index and stomatal traits (Fig. 3.34).

3.4.6 Expression Analysis of CaRab Genes

The gel images showed the bands of variable sizes, compared with 1 kb ladder. CaRab genes CaRab-A2-210, B-194, C-88, D-146, E-165, and H-197 exhibited expected lengths of amplicons (bp) (Fig. 3.35).

qRT-PCR was used to quantify the expression of selected genes in chickpea leaves from different treatments (C, S, and S+PGPR). The expression level of CaRab genes in clades A2, B, C, D, E, and H were assessed. When stressed plants were inoculated with *B. tequilensis*, gene expression was increased. Under the influence of salt stress, the expressions of these genes were down regulated in plants. CaRABA2 gene had the highest level of expression (Fig. 3.36). It was observed that RabB, -C, –D, and RabE, –H had essentially comparable expression patterns.

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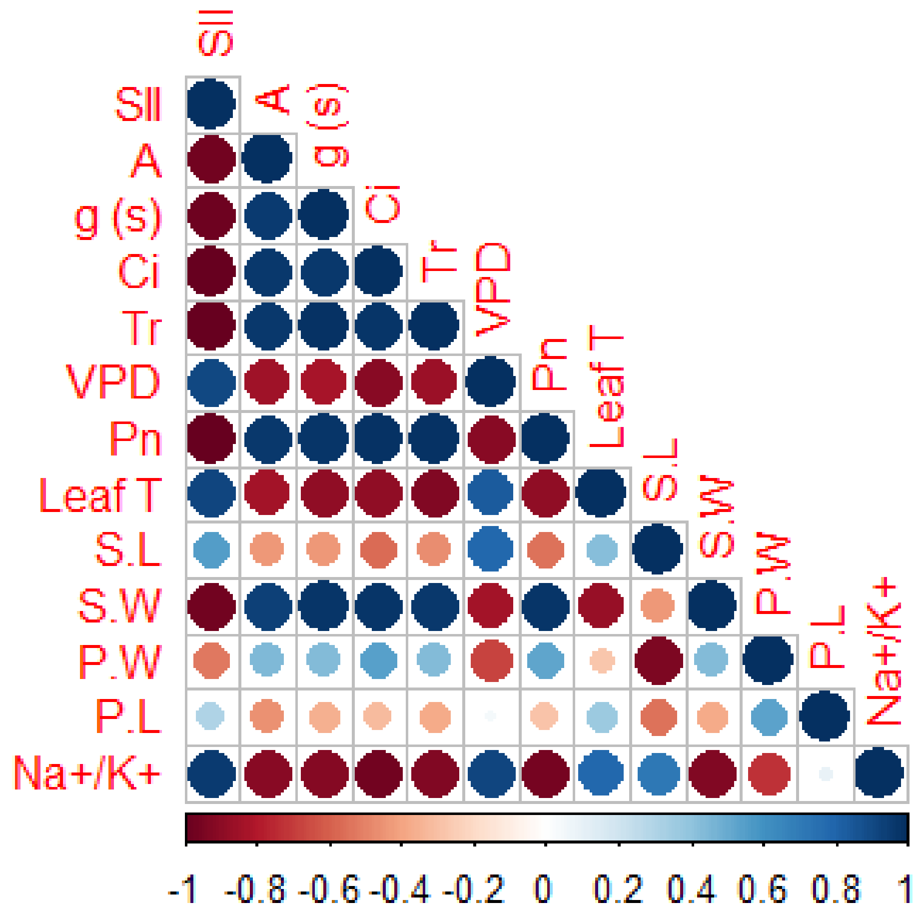

Fig. 3.34 Correlogram depicting the correlation between various studied parameters including SII (salt injury index), A (CO_2 assimilation, g (s) (stomatal conductance), Ci (intracellular CO_2 concentration), Tr (transpiration rate), VPD (vapor pressure deficit), Pn (net photosynthesis rate), Leaf T (leaf temperature), SL (stomatal length), SW (stomatal width), PW (pore width), PL (pore length), Na^+/K^+ (sodium potassium ratio).

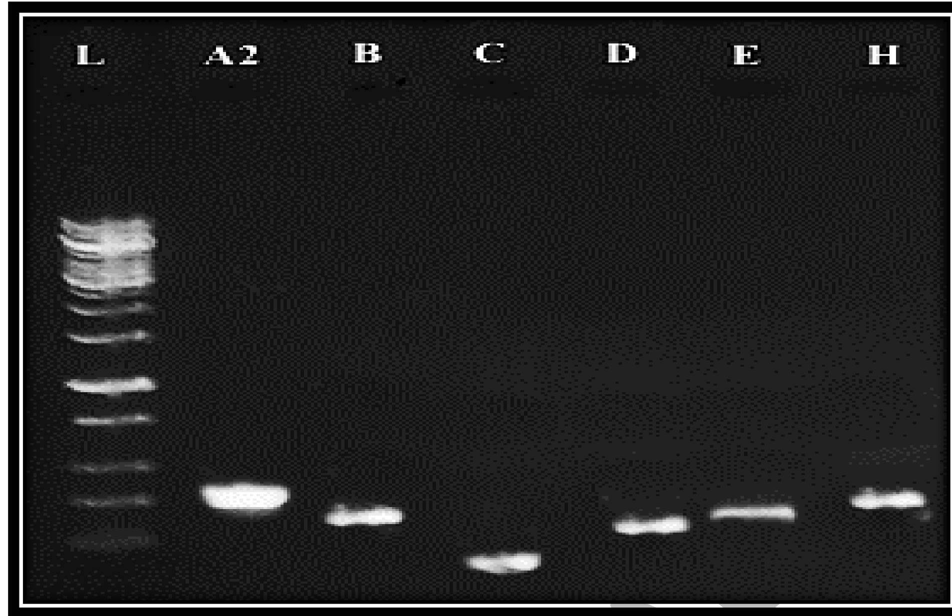

Fig. 3.35 RT-PCR products for six CaRab gene from bacterial treated plants under the influence of 100 mM NaCl). For amplicon size determination, a 1 kb DNA ladder was employed.

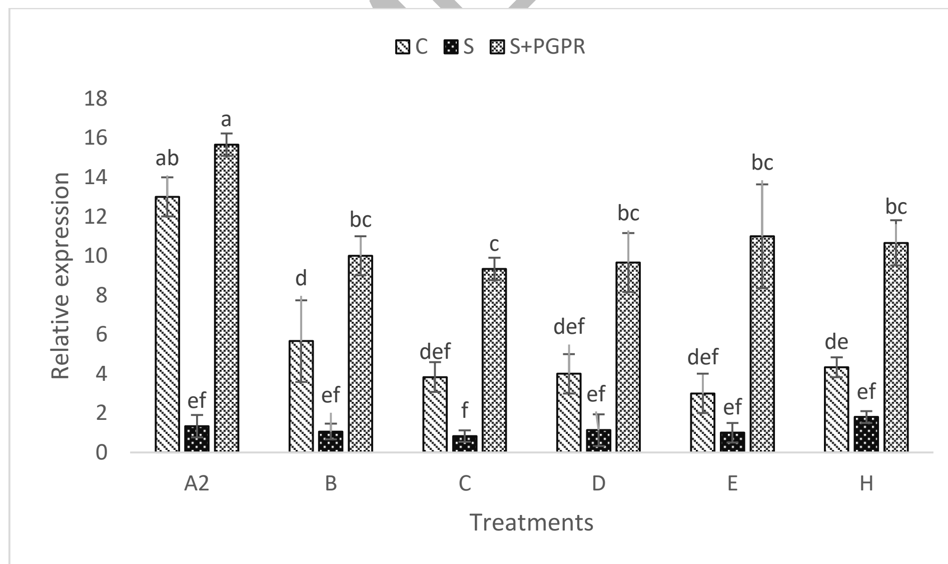

Fig. 3.36 Relative expression of CaRab genes, in the leaves of chickpea plants. Relative expression was observed in plants grown without PGPR and salt stress (C), plants grown under salt stress (S), and plants grown under salinity stress and inoculated with *B. tequilensis*. Standard errors are represented by bars. Tukey's least significant difference (HSD) test shows that dissimilar alphabets have statistically different values ($P<0.05$) from each other.

3.4.7 Linear Regression Analysis between Sodium Accumulation and Expression of CaRab Genes

The link between CaRab gene expression and the Na^+/K^+ ratio salinity stress alleviation in different treatments was investigated further, using linear regression analyses. The Na^+/K^+ ratio was computed as a function of the expression of all six genes. The expression of all genes, as well as the expression of individual genes was compared with Na^+/K^+ data. The Na^+/K^+ ratio in leaves and the expression profile of CaRabA2 had the strongest correlation (R2 = 0.6615, p<0.01) (Fig. 3.37). All other genes had a correlation coefficient (R2) of less than 0.5 with Na^+/K^+ ratio.

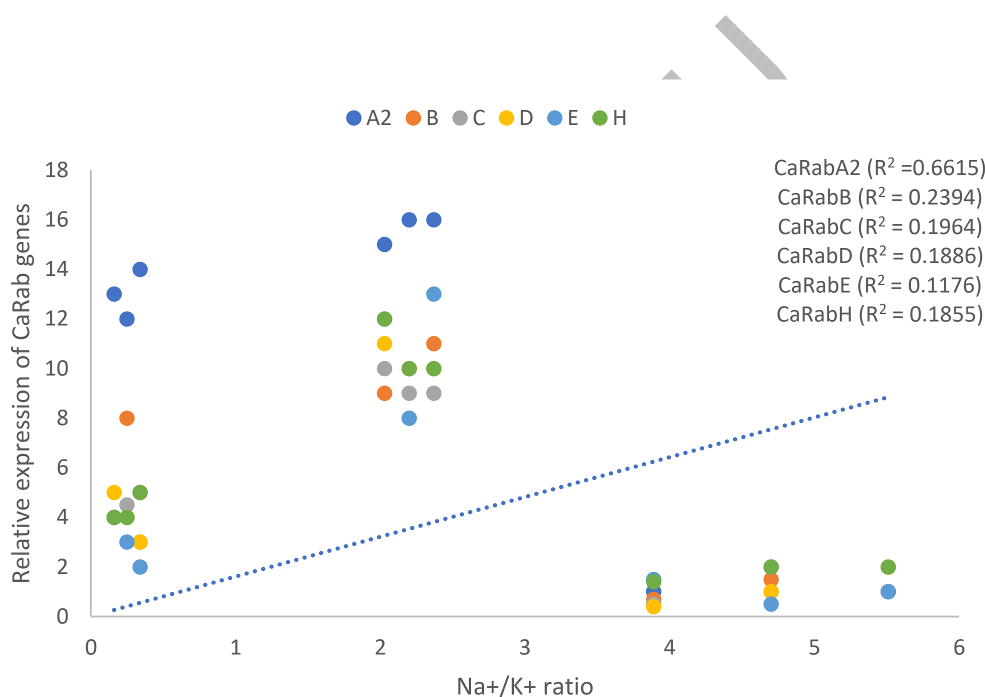

Fig. 3.37 Na^+ buildup in leaf samples and linear regression of six CaRab gene expression patterns, displayed as a Na^+/K^+ ratio. Pearson's correlation analysis was performed using Microsoft Excel. For clarity, just line of CaRabA2 of the best fit is displayed.

4. DISCUSSION

The main yield-limiting abiotic stress is salinity. It has a negative impact on the biochemistry, structure, and function of plants and soil (Hasanuzzaman *et al*., 2017). Plant health have been proven to be negatively impacted by salinity, and many scientists are striving to counteract these consequences. For the last few decades, use of salt tolerant microorganisms has become very popular. The assortement, screening, and inoculation of various stress tolerant PGPB for the improved performance of conventional farming would be expressly necessary to overcome the issues of global environmental change, low crop production, and raising food demand (FAO, 2018). Furthermore, the harmful impacts of organic composts and pesticides can be diminished with the help of these systems.

Halotolerant bacteria were initially discovered in the rhizospheric soil of *C. murale*, *J. adhatoda*, and *C. murale* that were obtained from Khewra salt mine in Pakistan. Khewra is famous for its salt reserves, and it is the second biggest salt mine of the world. From the collected rhizospheric soil, six highly salt tolerant bacterial strains were selected. Their sequence analysis recognized these isolates as *S. pasteuri*, *B. megaterium*, *B tequilensis*, *B. xiamenensis*, *P. aeruginosa* and *P. putida*. Previous studies have also described the isolation of various species of *Pseudomonas, Bacillus, Staphylococcus, Enterobacter, Virgibacillus, Terribacillus* and *Halobacillus* etc, from the same region (Roohi *et al*., 2012). Arora *et al*. (2014) had also isolated halotolerant *Bacillus spp.* from coastal region of Gujrat. The antibiotic resistance of these bacterial strains depicts their ability to persist in the challenging rhizospheric environment (Xia *et al*., 2020). Antibiotic resistance may vary due to morphological and genetic diversity differences among these strains.

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The capacity of the most efficient salt-tolerant bacterial strains to stimulate plant growth and manufacture several extracellular enzymes was tested, next. Plant growth and colonization are aided by the indole acetic acid production (Etesami *et al*., 2015). IAA has been found to be produced by a variety of *Azotobacter*, *Bacillus*, and *Pseudomonas* species (Verma *et al*., 2018). The findings of this inverstigation showed that all the chosen strains can produce IAA and can be employed as PGPB. The production of HCN and siderophore are the indirect PGP traits and these were also found among the isolates of the current

study. Bacterial strains *B. tequilensis* and *P. putida* were able to produce HCN. Siderophores can bind to ferric ion to make it available for plant uptake; that's why they are called as iron chelators. Siderophore can be produced by a variety of rhizospheric bacteria (Priyanka *et al*., 2017). According to Abdelshafy *et al*. (2020), halotolerant *Bacillus sp.* and *Pseudomonas sp.* can produce siderophore and IAA.

In both the presence and absence of tryptophan, all of the strains in the current study were able to synthesize indole acteic acid. Due to more production of indole-3-acetic acid, *B. tequilensis* was deemed the most promising strain. *Pseudomonas mendocina*, cultured from salt-affected soil, had previously demonstrated the ability to generate IAA (Sudhir *et al*., 2009). IAA production is possible in almost all *Bacillus* strains, with or without the addition of Tryptophan (Chagas *et al*., 2015). The bacterial strains were also efficient in the production of other hormones like GA3 and ABA. However, their production was reduced in the presence of tryptophan, in culture media. The reason might be the incompatible interaction of GA3 and ABA with tryptophan (Naz and Bano, 2010). Their findings also suggested that the release of diverse explosive biomolecules by bacterial isolates is linked to the secretion of phytohormones by the PGPR and both may serve as biological indicators of different biochemical processes. To test the influence of pH and temperature, three bacterial strains with the best PGP properties were used. The maximum growth of *B. megaterium* and *P. putida* was detected at pH 7, while the best growth of *B. tequilensis* was observed at pH 8. These results have been validated by Wen *et al*. (2009). Rohban *et al*. (2009) also reported excellent growth of salt tolerant bacterial isolates at pH 7-9 and 28-37 °C temperature. Under salinity stress, certain bacterial strains were found to have a favorable influence on wheat growth, *in vivo*. Chakraborty *et al*. (2011) have also observed better growth of *Vigna radiata, Cicer arietinum* and *Oryza sativa* by the inoculation of *Bacillus cereus*. Due to the inoculation of PGPR, efficient uptake of NPK have also been documented earlier (Xiaohui *et al*., 2017). The growth of the plant is aided by PGPR under variable environmental stresses (Dimkpa *et al*., 2009).

The second part of the study has validated the efficiency of rhizobacteria in inducing salinity tolerance by promoting the growth of plant. PGPR has evolved several methods to deal with varied stresses. Active salt tolerant ACC deaminase producing

microbes are used to improve stress tolerance and plant yield by the bio-augmentation of seeds (Ilangumaran and Smith, 2017). The ACC deaminase is produced as root exudate, converts to -ketobutyrate and ammonia and results in the production of ethylene, which has a substantial influence on plant development and function in stressful situations (Zhang *et al*., 2018). The selected bacterial strains efficiently produced ACC deaminase. It has previously been reported that Bacillus strains producing ACC deaminase efficiently improved the growth of wheat seedlings under salinity stress conditions (Din *et al*., 2019). Exopolysaccharides help plants retain moisture and grow under stressful conditions. Exopolysaccharides offer protection during dehydration, plant-microbe interaction, microbial accumulation, surface attachment, and bioremediation (Naseem *et al*., 2018). The findings of the current analysis showed an increased production of EPS under salinity stress and suggested the protective role of halotolerant bacterial strains (Li *et al*., 2017). IAA improves PGPR growth and colonization by increasing the outflow of plant root secretions, which quickly fill in as an energy source for them (Etesami *et al*., 2015). Auxin producing bacteria have previously been shown to improve root development and nutrient uptake, and assisting plants in coping with saline condition (Yasin *et al*., 2018). Auxin is synthesized by a variety *of Bacillus, Azotobacter, and Pseudomonas* species (Cassán *et al*., 2014).

The current study found that all the tested strains can produce IAA, indicating that they could be used as PGPR. Phosphate solubilizing bacteria not only provide phosphorus to plants, but they also help them grow by improving nitrogen fixation, enhancing trace element accessibility, and creating plant hormones (Kumar *et al*., 2013). PGPR also promotes plant growth and reduces salt stress by producing the phytohormones and increasing the intake of nutrients. The drastic effects of synthetic fertilizers on crop productivity have also been reduced by PGPR (Kumar *et al*., 2015). All of the chosen bacterial isolated tested positive for the ability to solubilize phosphate, which is an important plant growth promoter. Our results depicted that among all the studied bacterial strains, *B. tequilensis* produced the highest levels of ACCD, EPS and IAA. *B. tequilensis* also exhibited efficient phosphate solubilizing ability and proved it to be the best PGPR.

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In this study, seed treatment with PGPR raised germination percentage and improved a variety of wheat plant growth characteristics. Both sugar and proline content were increased in bacterial infected wheat seedlings, cultivated under saline conditions. As a result, by enhancing metabolic resistance mechanisms, PGPR inoculants managed to improve growth of plants under varying levels of salinity stress (Ilangumaran and Smith, 2017).

Salinity stress considerably reduced plant chlorophyll content, but these contents were increased with bacterial inoculation. Salinity stress increase photo-oxidation to decrease the concentration of chlorophyll a and b (Rahdari *et al*., 2012). Scientists have previously suggested that under salt stress circumstances, bacterial inoculation in plants produces more photosynthetic pigments (Sapre *et al*., 2018). Under both stressful and non-stressed conditions, carotenoid content was considerably raised due to bacterial application. Carotenoid are important for the breakdown of singlet oxygen and high carotenoid concentration is associated with genotype tolerance (Efeolu *et al*., 2009).

Salinity results in osmotic stress and results in the reduction of RWC (Fahad *et al*., 2015). Our findings revealed that plants treated with PGPR had higher RWC, whereas sick plants had lower RWC. Under stressful conditions, a drop in RWC has been reorted earlier (Dekov *et al*., 2000; Nayyar and Gupta, 2006). Under stressful conditions, relative electrolyte leakage was increased and resulted in an increase in POD and CAT activity. Among all treatments, bacterial inoculation diminished the adversity of stress by decreasing electrolytic leakage. Previously, *Bacillus sp.* has also been reported to decrease electrolytic leakage and imparting membrane stability (Vardharajula *et al*., 2011). The results of present study showed that STI of wheat seedlings was significantly reduced in salt stress condition. However, PGPR inoculants showed elevated STI value, correspondingly. Previous studies have also reported increased STI value in *Capsicum annum* (Yasin *et al*., 2018). Following PGPR inoculation under salt stress, all three inoculated bacteria showed significant increases in growth attributes of plants. The use of PGPR has been shown to boost the length of root shoots and overall plant vigor (Farooq and Bano, 2013)., Karlidag *et al*. (2013) demonstrated enhanced growth features due to bacterial inoculation, under the influence of salinity stress.

When PGPR-treated wheat plants were compared to un-inoculated control plants, antioxidant enzyme activities (SOD, POD, and CAT) were significantly increased. Our findings are consistent with previous findings (Hmaeid *et al*., 2019), who discovered elevated levels of ROS scavenging enzymes in *Sulla carnosa* inoculated with PGPR. under saline stress. Under the influence of excess salt, plants start producing excessive level of MDA. The results of this study depicted the same. Bacterial inoculation reduced MDA levels in wheat plants, and improved stress tolerance. The outcomes of the present study are comparable to the outcomes of Singh and Jha (2017), who also described a decrease in MDA production in *S. maltophilia* SBP-9 inoculated wheat plants, under salinity stress. H_2O_2 level was increased in the plants under salt stress, but bacterial inoculation decreased its production. PGPR has been well documented to lower the levels of lipid peroxidation, superoxide anions and H_2O_2 by stimulating defense mechanisms (Gupta *et al*., 2017).

Ionic stress at cellular level causes negative impacts on crop yield and production. In plants, numerous genes that are upregulated under stress conditions have been stated to have a role in numerous metabolic pathways and they regulate the mechanism of transcription, signal transduction and ion transport (Deinlein *et al*., 2014). Salt overlay sensitive (SOS) pathways and various ion transporters help in the alleviation of ionic stress in plants (Zhu, 2000). SOS1 is an important plasma membrane sodium and hydrogen ions antiporter that helps plants to cope with salinity stress. Pyridoxal-5-phosphate is an important cofactor for various enzymes. Its synthesis is regulated by pyridoxal kinase that is encoded by SOS4 gene. Moreover, SOS4 gene is also related with the production of IAA (Mahajan *et al*., 2008). The SOS protein family is clearly shown to be able to mediate salt tolerance directly and indirectly (Ramezani *et al*., 2013). The findings of the current research showed upregulation of SOS genes in plants inoculated with halotolerant *B. tequilensis,* under salinity stress and described their possible role in combating with salinity. It has previously been reported that the rice SOS genes play a significant role in the adaptive mechanism to salinity tolerance (El Mahi *et al*., 2019).

The third part of this study was designed to characterize, identify, and assess the influence of *B. tequilensis* on salinity tolerance and growth promotion of chickpea. This research will aid in the application of PGPR to improve plant tolerance in stressful

environments, particularly salinity, and to promote the growth of chickpea plant. *B. tequilensis* exhibited bacterial flocculation and biofilm formation traits. Bacterial flocculation has been directly related to the production of bacterial exopolysaccharides. It aids bacterial existence in stressed environments and assists plant in stress tolerance (Qureshi and Sabri, 2012). Exopolysaccharides are associated with the formation of a bacterial biofilm, which facilitates bacterial adhesion on plant root (Chen *et al*., 2013). High floc yield protects host plants at higher salt concentrations (Hong *et al*., 2017). Furthermore, under salt stress conditions, biofilm serves as a barrier between both bacteria and surroundings and safeguard them, inside the EPS layer. This study revealed maximum biofilm formation at higher salt concentration. Kasim *et al.* (2016) previously stated that the increasing concentrations of NaCl enhance the formation of biofilms. Salt-tolerance features of studied microorganism were further supported and explained by SEM analysis. Bacterial cells can collaborate with plant root system to improve its water retention and stress tolerance. FTIR spectroscopy revealed the presence of hydroxyl, amino and carboxyl groups, which binds with Na^+ ions and provide tolerance against salinity (Watanabe *et al*., 2003; Nunkaew *et al*., 2015). In this study, the amplification of 1-aminocyclopropane-1-carboxylate deaminase (acdS) and pyrroloquinoline quinone (pqqE) genes described the growth promoting ability of *B. tequilensis.* Gene acdS promotes the formation of ammonia and alpha ketobutyrate from ACC, leading to lower ethylene production and promotes plant development and growth (Kang *et al*., 2019). Gene pqqE is a key element of the PQQE operon and is engaged in phosphate solubilization (Hayat *et al*., 2010).

Inoculation of *B. tequilensis* improved physiological traits of chickpea seedlings, in this study. PGPR has been described to improve germination of different plants (Nelson, 2004). During salinity stress, increase in RWC has also been described earlier (Rakshapal *et al*., 2013). Under the stress condition, electrolytic discharge (such as potassium ions) increases by replacing calcium ions present in plasma membrane. As a result, membrane permeability is compromised, resulting in increased electrolyte efflux within plant cells/tissues (Garg and Manchanda, 2009). In comparison to control treatments, plants under the impact of *B. tequilensis* and salinity stress displayed reduced electrolytic leakage. Chlorophyll content was shown to be lower under salt stress

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conditions, according to the results of this study. This might be because the chlorophyllase, which is salinity stimulated and degrades pigment proteins that decreases the synthesis of chlorophyll level in plants (Abd Allah *et al*., 2018). Under salinity stress, carotenoids synthesis was increased in chickpea. Carotenoids act as antioxidants to manage stressful conditions (El Esawi *et al*., 2019).

For stress management, plant growth promoting rhizobacteria stimulates the production of ROS scavenging enzymes. In the present investigation, under the effect of salt stress, *B. tequilensis* enhanced the production of SOD, POD, and CAT. It has previously been observed that when exposed to high levels of salt stress, antioxidant enzyme activities are increased, allowing damaging free radicals to be eliminated (Abd Allah *et al*., 2018; Rasool *et al*., 2013). Increased level of POD results in lignin synthesis and helps in stress tolerance (Boerjan *et al*., 2003). Moreover, the increased production of SOD, POD and CAT protects chloroplast and other organelles of plants where important metabolic processes occurs (Han and Lee, 2005; Hashem *et al*., 2016). CAT activity also lowers the production of hydrogen peroxide (Mutlu *et al*., 2009). Oxidative damage under stress is indicated by lipid peroxidation that is dignified by MDA production in plants. In the current investigation, it was observed that *B. tequilensis* inoculation decreased the production of MDA, even under varying levels of salinity. The production of H_2O_2 was high in chickpea seedlings under saline conditions but the inoculation of *B. tequilensis* stimulated defense mechanism and lowered its production (Gupta *et al*., 2017). This study has linked the bacterial production of exopolysaccharides with increased STI of chickpea seedlings. Exopolysaccharides may alter chickpea rhizosphere by forming a biofilm on root surface, resulting in improved water and nutrient availability (Hussain *et al*., 2014).

In the fourth part of the present study, the effects of salt-tolerant *B. tequilensis* on gas exchange parameters, stomatal characteristics, leaf elemental analyses, and osmotality of chickpea were examined. After plants were treated with *B. tequilensis*, stress levels were reduced under the same salinity regime. Osmotic stress and ion cytotoxicity are the main causes of salinity stress in plants (Shabala and Mackay, 2011). Crop productivity under salinity stress is achieved by combining the distributions of restricted photosynthetic carbon gain between reproduction and growth. Photosynthesis is a complex process that

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depends on the availability of water carbon dioxide and light (Wong *et al*., 1979; Farquhar and Sharkey, 1982). Decreased CO_2 intake becomes the significant constraint in photosynthesis after NaCl-induced stomatal closure, resulting in an increase in leaf temperature (Chaves *et al*., 2009). In sorghum, wheat, and barley, salt stress significantly reduced CO_2 assimilation and stomatal conductance (Jiang *et al*., 2006; Zheng *et al*., 2009; Yan *et al*., 2012). However, the treatment of *B. tequilensis* increased gas exchange characteristics of chickpea under salinity stress, resulting in chickpea tolerance to salinity stress.

In plants, stomata are essential for regulating gaseous exchange. Stomatal response of plants was also altered by high salt levels. In comparison to control plants, plants treated with high salt (100 mM NaCl) showed relatively closed stomata. Salt stress inhibits water flow, which lowers leaf water potential and encourages stomata closure (AzevedoNeto *et al.,* 2004). Plants treated with *B. tequilensis* and salt, on the other hand, had open and normal stomata. The normal size of stomata is due to microorganisms maintaining plant water status while lowering root hydraulic conductivity (Mushtaq *et al*., 2004).

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Leaf necrosis is one of the first signs of NaCl poisoning (Yin *et al*., 2010). Plants were badly harmed in the proposed investigation due to salt stress. The salt damage index was lowered in saline circumstances after inoculation with *B. tequilensis*. On the leaves, the effect of *B. tequilensis* in ameliorating salt stress was clearly visible. The severe abscission of leaves has previously been shown to be one of the principal reactions of plants under extreme salt conditions (Gómez-Cadenas *et al*., 2002). The significant abscission of leaves under salt stress was detected in our results, however it was mitigated after PGPR inoculation. Under salt stress, the sodium ions content of chickpea leaves increased. This increase in Na+ content may be the primary cause of plant salt stress damage (Shrivastava and Kumar, 2015). Excessive ionic flux and electrolyte imbalance caused by salt stress can also cause membrane damage. It displaces membrane-associated calcium ions and alters cell membrane permeability (Kang *et al*. 2014). The potassium ion level was found to be lower since it has been proven over the last five decades that the concentration of sodium salts in the soil prevents potassium absorption, causing plants to suffer from K^+ shortage (Abu-Al-Basal *et al*., 2009).

Expression of six selected CaRab genes was validated using qRT-PCR. The function of the vesicular trafficking machinery in the modulation of plant responses to a variety of abiotic stressors and it has been extensively established, over the last decade (Tripathy *et al*., 2021). The expression of CaRabA2 was found to be higher than other genes. RabA genes are implicated in plant responses to abiotic stressors (Ambastha *et al*., 2021). Under the effect of *B. tequilensis* and salt stress, the expression of RabB, -C, –D, and RabE, –H was nearly identical, indicating their function as a part of a collaborative network. The co-regulation of these clades could be due to their roles in vesicle trafficking from the rough endoplasmic reticulum and golgi bodies to plasma membranes.

4.1 CONCLUSION AND FUTURE PROSPECTS

Our study revealed that the selected three bacterial strains (*Bacillus megaterium, B. tequilensis* and *Pseudomonas putida*) can tolerate higher concentrations of salt and promote plant growth traits. These strains stimulated plant growth promotion and induced salinity tolerance by the secretion of various hormones and extracellular enzymes. This is the first comprehensive study in which three halotolerant ACCD and EPS producing PGPRs have been studied on wheat and chickpea seedlings, simultaneously. Detailed results indicated the best performance of *B. tequilensis* in growth promotion of wheat and chickpea seedlings and salt stress tolerance. This bacterium helps plants to tolerate salinity stress conditions by enhancing the production of total soluble sugar, proline and antioxidant enzymes. This bacterium can be further used as bioinoculant to tolerate salinity stress in different crops. When compared to salt-affected circumstances, results demonstrated that inoculating chickpea with *B. tequilensis* under salinity stress considerably improved gas exchange metrics, stomatal and ionic characteristics. Furthermore, the highest correlation between salt buildup and CaRabA2 gene expression suggests that it is involved in chickpea salinity tolerance. In the future, we may be able to use transformation to overexpress CaRabA2 genes in plants to improve their tolerance to extreme salt stress.

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APPENDIX

Appendix 1 FASTA sequences of selected halotolerant bacteria.

>MPP1

CCTTCGACGGCTAGCTCCATAAATGGTTACTCCACCGGCTTCGGGTGTTACAA
ACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCG
TAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGC
AGACTACAATCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGG
TTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
TCAACTTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGC
ACCACCTGTCACTTTGTCCCCCGAAGGGGAAAACTCTATCTCTAGAGTGGTCA
AAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
GCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGG
TCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCG
GAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTA
TCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCA
GAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCT
ACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGA
CCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTAC
GCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCG
CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATCAGGTACCGTCAAGAC
GTGCACAGTTACTTACACGTTTGTTCTTCCCTGATAACAGAGTTTTACGATCC
GAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCG
GAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCA
GTGTGGCCGATCACCCTCTCAGGTCGGCTACGTATCGTTGCCTTGGTAAGCCG
TTACCTTACCAACTAGCTAATACGGCGCGGATCCATCTATAAGTGACAGCAA
GGCCGTCTTTCACTATTGAACCATGCGGTTCAAAATCTTATCCGGTATTAGCT
CCGGTTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTTATCCACGTGTTACTC
ACCCGTCCGCCGCTAACGTCAAAGGAGCAAGCTCCTTATCTGTTCGCTCGACT
TGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGTTCCCAACTC

>MPP7

GCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATG
ACGTGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGG
ATAACTTCGGGAAACCGAGGCTAATACCGGATAGGATCTTCTCCTTCATGGG
AGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTAGGGAAGAACA
AGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA

ATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAAGTGCAGAAGAGA
AAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA
CCAGTGGCGAAGGCGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
GGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGG
GACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGT
TGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGAT
GGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTC
TCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCGAAGTCGGTGGAGTAACCGTAA
GGACGTAGCCGCCTAAGGTGGGACAGATGATTGGGGT

>MPP8

TGGCGGCGTGCCTAATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCC
TGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCA
TGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGG
CGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGG
GAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGA
TGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTG
AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGACGGTCGCAAGACTGAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATA
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Appendix 2 Growth of wheat seedlings under different treatments. (A) Seedlings under salinity stress, (B) Seedlings under the influence of *B. megaterium* and salinity stress, (C) Seedlings under the influence of *B. tequilensis* and salinity stress and (D) Seedlings under the influence of *P. putida* and salinity stress.

Appendix 3 Effect of treatments on growth of wheat seedlings.

Appendix 4 Effect of different treatments on growth of chickpea seedlings after 21 days of sowing.

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Employment of Halotolerant Plant Growth Promoting Bacteria to Mitigate Soil Salinity in Wheat and Chickpea by Urooj Haroon .

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Isolation of Halotolerant Bacteria from Rhizosphere of Khewra Salt Mine Halophytes and their Application to Induce Salt Tolerance in Wheat

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ABSTRACT

Broad manipulation of halotolerant plant growth promoting bacteria (PGPB) is very important in agriculture. In the present research, survival and adaptability of PGPB was determined under saline conditions. From the rhizospheric soil of *Justicia adhatoda*, *Chenopodium murale* and *Cenchrus ciliaris*, growing in Khewra salt mine, 20 bacterial strains were isolated. Out of these, six strains were found to be highly salt tolerant and they were characterized on the basis of their morphology, biochemical traits and sequence analysis of 16S-rRNA gene. These strains were identified as *Bacillus megaterium*, *Bacillus tequilensis*, *Bacillus xiamenensis*, *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Staphylococcus pasteuri*. On the basis of plant growth promoting activities and extracellular enzyme secretions, *B. megaterium*, *B. tequilensis* and *P. putida* were selected and used for further analyses. All selected PGPRs showed antibiotics tolerance and the maximum tolerance was exhibited by *B. tequilensis*. Moreover, all the selected bacterial strains produced various phytohormones including indole-3-acetic acid (IAA), gibberellic acid (GA3) and abscisic acid (ABA). These strains enhanced root length, shoot length and leaf area of wheat seedlings by increasing the uptake of macronutrients (nitrogen, phosphorus and potassium). On the basis of these findings, these PGPRs can be considered as potential bio-inoculants to mitigate salt tolerance.

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KEYWORDS
Plant growth-promoting bacteria; rhizospheric soil; phytohormones; salt tolerance; wheat

Introduction

In agriculture, salinity is a condition that refers to the abundance of salts above the normal level required by the plant (Yadav et al. 2011). Salinity is extremely dangerous and is also called soil cancer (Chandio et al. 2017). This phenomenon adversely affects the production of various crops. Typically, salinity can be distinguished by the formation of white crust over the soil surface like snow covering the surface of the soil. The formation of a white-colored crust represents the amount of sodium present in it (Sonon et al. 2015).

Salinity is a major abiotic stress and it restricts the growth and efficiency of plants in different areas of the world. Salts can easily bind with water and decrease the availability of free water in the soil. The utilization of low-quality saline water is toxic for plants and deteriorates soil structure and texture. Soil salinity is extensively highlighted as the key agricultural problem due to its severe influence on the growth and development of the plant. Salt stress is the chief environmental issue that restricts the global output of cereal crops and beneficial trees, specifically in developing countries (Boyer 1982). In the world, ~20% of land eligible for cultivation is saline (Sheng et al. 2008).

increasing population. To improve the crop and aquaculture production under various abiotic stresses such as salinity, plants are intrinsically outfitted with stress tolerance ability to react to particular stress. To cope with the salt stress, plants adopt various mechanisms including ion exchange, hormone stimulation, reactive oxygen species (ROS) scavenging, and activation of signaling mechanisms to survive under stressed conditions. Increasing the production of prime foods is an absolute necessity for humans. It is, consequently, essential to produce salt-tolerant plants for planting in saline lands (Zhu 2000; Wang et al. 2003).

Some plant growth-promoting bacteria (PGPB) have well-defined mechanisms that play an important role in salinity stress tolerance and promote the growth of plants. So, it is required to explore the elite and meticulous type of organism including microbes that have the potential to mitigate salinity and improve crop productivity. Prokaryotes are able to acclimatize an extensive variety of ecological environments, like extremes of temperature, pressure, salinity, pH, radiation, etc. (Nath and Bharathi 2011). The plant growth-promoting rhizobacteria *Serratia marcescens* alleviate salinity resistance in wheat (Singh and Jha 2016). Plant

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Halotolerant Plant Growth-Promoting Rhizobacteria Induce Salinity Tolerance in Wheat by Enhancing the Expression of SOS Genes

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Abstract
Soil salinity is one of the main yield-limiting factors in various crops. Under different environmental stresses, many rhizobacteria have demonstrated encouraging role in enhancing plant growth and tolerate stress conditions. In this study, three potential 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase- and exopolysaccharides (EPS)-secreting bacterial strains including *Bacillus megaterium*, *B. tequilensis*, and *Pseudomonas putida* have been assessed for their growth-promoting characteristics. These bacterial strains positively affected the physiology, biochemistry, and antioxidant enzymatic activities of wheat plant, under salinity stress. Results of this study depicted that the inoculation of PGPR positively invigorates growth attributes like relative water content and photosynthetic pigments of wheat seedling under saline conditions. Moreover, plants inoculated with PGPR also showed decreased concentration of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Inoculation of PGPR reduced electrolytic leakage and enhanced enzymatic activity for the scavenging of reactive oxygen species (ROS). These PGPR also increased the production of proline and total soluble sugar. Expression analysis of selected genes by qPCR revealed higher expression of Salt Overly Sensitive (SOS1 and SOS4) genes and predicted their potential role in stress tolerance. These genes can be further overexpressed in wheat plant to tolerate salinity stress. On the basis of these findings, it can be concluded that the priming of seeds with aforementioned PGPR can decrease the adverse effects of salinity on wheat plant.

Keywords Soil salinity · ACC deaminase · Exopolysaccharides · PGPR · Wheat

Introduction

Numerous environmental stresses such as water deficiency, flooding, salinity, and extreme temperature conditions can affect the growth and development of plants and ultimately reduce their yield (Bano and Fatima 2009; Jha et al. 2011). Among these abiotic stresses, soil salinity is one of the most prominent abiotic stresses that restrict crop productivity (Munns and Gilliham 2015). Pakistan has mainly calcareous soils and salinity affects the overall yield of a variety of crops (Shrivastava and Kumar 2015). It has been reported that 6% of the cultivated land in Pakistan is highly saline (Ilangumaran and Smith 2017). More than 20% of cultivated areas, across the globe, have been reported to be under the influence of salt stress and this percentage is increasing, rapidly (Ruan et al. 2010; Lakhdar et al. 2009).

In dry and semi-dry areas of the world, soil salinity adversely disturbs the production of crops. It restricts the growth and production of crops by the generation of reactive oxygen species (ROS), sodium and chloride toxicity, and osmotic and nutrient imbalances (Mishra et al. 2013). Elevated level of ROS under various stresses can threat plant cells by oxidizing proteins, damaging DNA and RNA, doing lipid peroxidation, activating apoptosis, and inhibit-

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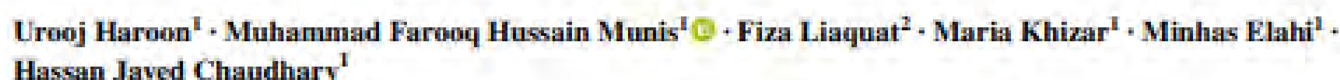
RESEARCH ARTICLE

Biofilm formation and flocculation potential analysis of halotolerant *Bacillus tequilensis* and its inoculation in soil to mitigate salinity stress of chickpea



Urooj Haroon[1] · Muhammad Farooq Hussain Munis[1] · Fiza Liaquat[2] · Maria Khizar[1] · Minhas Elahi[1] · Hassan Javed Chaudhary[1]

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Abstract Application of beneficial microbes in soil is an important avenue to control plant stresses. In this study, the salinity tolerance of halotolerant bacteria (*Bacillus tequilensis*) was investigated and the bacterium was inoculated in the soil to mitigate salinity stress. The results revealed the highest floc yield and biofilm formation ability of *B. tequilensis* at 100 mM NaCl concentration. Fourier transformed infrared spectroscopy depicted the presence of carbohydrates and proteins which binds with sodium ions (Na^+) and provide tolerance against salinity. Using PCR, plant growth-promoting bacterial genes viz., 1-aminocyclopropane-1-carboxylate deaminase and pyrroloquinoline quinone were successfully amplified from the genome of *B. tequilensis*. In the saline soil, *B. tequilensis* was inoculated and chickpea plants were grown. The bacterial strain improved the physiology, biochemistry, and antioxidant enzyme activities of the chickpea plant under salt stress. Plants inoculated with *B. tequilensis* exhibited higher relative water content, higher photosynthetic pigments, lower levels of hydrogen peroxide (H_2O_2) and malondialdehyde, and improved enzymatic activity for the scavenging of reactive oxygen species. The findings of this study suggest the sustainable use of *B. tequilensis* to mitigate the salinity stress of chickpea and other crops. This bacterium not only helps in the alleviation

Keywords Salinity · FTIR · acdS · pqqE · ROS

Introduction

Crop production is seriously hindered by the adverse effects of abiotic and biotic stresses. Plants are immobile and they are exposed to a range of challenges including water stress, heavy rains, alkalinity, temperature extremities, metal stress, nutrient depletion, phytopathogen and insect attacks. Among many environmental pressures, the extreme availability of dissolved salts (soil salinity) is one of the most significant issues, leading to decreased plant growth and compromised crop yield (Gupta and Pandey 2019). Around 7% of the total land and 20% of the cultivated area is influenced by salinity. Plants use a variety of halotolerant mechanisms, such as the synthesis of polyamine and osmolytes, reduction of reactive oxygen species, ion transport, antioxidant defense mechanisms and compartmentalization, etc. (Shukla et al. 2012).

The word "rhizobacteria" was first used to describe the soil bacterial community in 1978 by Kloepper and Schroth. Rhizobacteria competitively penetrate plant roots, stimulate growth, and decrease the incidence of plant infections. These helpful rhizobacteria are referred to as plant growth-promot-