Studies to determine mechanism of salt tolerant plant growth promoting fluorescent pseudomonads in enhancing growth, yield and oil content of sunflower under different saline conditions

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

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(Enrolment No. 398/12)

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SCHOOL FOR ENVIRONMENTAL SCIENCES

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UTTAR PRADESH, INDIA

2021

DEDICATED

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MY DADA AND NANI

CERTIFICATE

This is to certify that the thesis titled "**Studies to determine mechanism of salt tolerant plant growth promoting fluorescent pseudomonads in enhancing growth, yield and oil content of sunflower under different saline conditions**" submitted by **Ms. Tahmish Fatima** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D) regulations-1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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विभागाध्यक्ष
पर्यावरणीय सूक्ष्मजैविकीय विज्ञान विभाग
बाबासाहेब भीमराव अम्बेडकर विश्वविद्यालय
(केन्द्रीय विश्वविद्यालय), लखनऊ, २२६०२५

DECLARATION

This is to certify that I have worked on the research thesis entitled "**Studies to determine mechanism of salt tolerant plant growth promoting fluorescent pseudomonads in enhancing growth, yield and oil content of sunflower under different saline conditions**". The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree. "The thesis is essential free from all kinds of plagiarism".

Place: Lucknow

Date: 23.12.2021.

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ACKNOWLEDGEMENT

"There are none worthy of worship besides You (Allah). Glorified are You. Surely I am from the wrongdoers."

(Qur'an 21: 87)

In the name of Allah, the Most Beneficent, the Most Merciful. First and foremost, I am thankful to **Allah Almighty**, for giving me sound health, abilities, courage, patience and strength to complete this thesis.

It is a genuine pleasure to express my deepest thanks to the person who gave me the opportunity to work on such an innovative idea, my supervisor **Prof. Naveen Kumar Arora**, Dean and Head, Department of Environmental Science, School for Earth and Environmental Sciences, BBA University, Lucknow. A profound sense of gratitude binds me to my guide and my path-finder, who continually and convincingly conveyed a spirit of adventure and excitement in regards to this research. In addition, he patiently read my texts, structured papers, taught me essential skills and most importantly believed in me until the completion of this work. Working under the supervision of such a talented-mind with an amicable and positive disposition, was a pleasant privilege and indeed a great experience.

I take this opportunity to sincerely thank **Honorable Vice Chancellor, Prof. Sanjay Singh** for providing me with the amenities and a congenial work environment, to come up with the best possible results.

I would like to acknowledge the **University Grants Commission** (UGC), **Maulana Azad National Fellowship**, (Ministry of Minority Affairs; MoMA), India, for providing financial support. Completion of this work was possible with the support of **University Sophisticated Instrumentation Centre** (USIC), BBA University, Lucknow, **Birbal Sahni Institute of Paleobotany,** Lucknow, **National**

Agriculturally Important Microbial Culture Collection (NAIMCC), Mau and **National Centre for Microbial Resources** (NCMR), Pune for extending instrumentation facilities.

I also want to extend my deepest gratitude towards **Prof. Ram Chandra** (Head, DEM), **Prof. Rajesh Kumar**, **Dr. Vinay Singh Baghel** (Assistant Prof.), **Dr. Jay Shankar Singh** (Assistant Prof.), **Dr. Ram Naresh Bhargava** (Assistant Prof.), **Dr. Pankaj Kumar Arora** (Assistant Prof.), **Dr. Digvijay Verma** (Assistant Prof.), **Dr. Harish Chandra** (Assistant Prof.) and **Dr. Ravi Kumar Gupta** (Assistant Prof.) of the Department of Environmental Microbiology for their support and cooperation.

Moving to my lab, let me thank my seniors **Late Dr. Rachna Singh, Dr. Maya Verma, Dr. Jai Prakash, Dr. Rishabh Chitranshi, Dr. Shamshad Ahmad** and **Dr. Gaurav Saxena** who provided insight to help me grow and succeed. Dedicating few lines to an amazing researcher, a beautiful soul and a helping senior **Late Dr. Rachna Singh,** would like to say that I was lucky enough to cross paths before you left for the final journey. You trusted me, you taught me when things were hard and I was losing, I miss you.

I am wholeheartedly thankful to my wonderful colleagues cum friends **Miss Isha Mishra, Mrs Renu Verma** and **Miss Sushma Verma** for caring and supporting me and the role you all played in my life is invaluable. My special thanks and tonnes of appreciation would never be enough for the favors, **Isha,** you did; I will always keep your kindness in my heart. I am also thankful to all my juniors for their help and for cheering me during the journey of this work.

I convey my sincere gratitude towards staff members **Ms. Naincy**, **Mr. Manish** and **Mr. Sarju Singh** for their continuous support and cooperation. **Naincy ma'am**, though we were good professionally, but best as friends. You helped me beat the

Morning blues through your laughter and most importantly, your wisdom gave me some important lessons of life, forever grateful.

"*Next to God, Thy Parents*", My mother (**Shamsa Talat**) and my father (**Dr. Iftekharul Haque**), you both didn't give up, you didn't give in and that is the reason I am standing here today. Dedicating my work to my late beloved grandfather and nani, who brought love, warmth and happiness to me but are unfortunately not here to witness my hardwork. My heartfelt thanks to my sister (**Mehvish Fatima**) and brother (**Usman Ul Haque**) for holding up my morals, praying and caring for me and the unwavering support and sacrifices. I am indebted to my entire family, my cousins **Noman Ul Haque**, **Adnan Ul Haque,** my brother-in-law **(Salman Haque)** and my two-little nieces (**Zehra and Zanirah**) for lightening up the heavy load. Special thanks to my cousin **Hamza Siddique,** without his help my field work would not have been a success. Friends are the stress-busters and no journey can become memorable without them. Thank you my dear friends, **Dr. Kanika Sharma, Dr. Shilpi Singh, Rifat Azam, Nazia Parveen, Asma Farooq** and **Priyanka Chaudhary.** Special thanks to my friend **Abdul Quadir Siddique** who put up with my odd hours, and provided me with lifts and emotional help.

Place: Lucknow

Date: 23-12-2021.

Tahmish Fatima.

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%	Percent
$(NH_4)_2SO4$	Ammonium Sulphate
°C	Degree Celsius
°E	Degree East
°N	Degree North
µg	Micro Gram
µL	Micro Liters
µS/cm	Micro-Siemens per Centimeter
µS/m	Micro-Siemens per meter
ACC	1-Aminocyclopropane-1-Carboxylate
ACCD	1-Aminocyclopropane-1-Carboxylate Deaminase
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Sequence Tool
BNF	Biological Nitrogen Fixation
bp	Base Pairs
BSA	Bovine Serum Albumin
BTB	Bromothymol Blue
CAS	Chrome Azurol S
CFU	Colony Forming Unit
cm	Centimeters
CMC	Carboxymethylcellulose
DAS	Days After Sowing
DF	Dworkin and Foster
DMRT	Duncan's Multiplicity Range Test
DNA	Deoxy Ribonucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
dS/m	Deci-Siemens per meter
EC	Electrical Conductivity
EDS	Energy Dispersive Spectroscopy
EL	Electrolyte Leakage
EPS	Exopolysaccharides
FT-IR	Fourier Transmission Infra-Red
g	Gram

GAs	Gibberellins
GC-MS	Gas Chromatography-Mass Spectrometry
GPA	Glucose Peptone Agar
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen Sulphide
ha	Hectare
HAM	Hofer's Alkaline Media
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium
hrs	Hours
IAA	Indole-3-Acetic Acid
ILSCM	Inverted Laser Scanning Confocal Microscopy
K	Potassium
kDa	Kilo Dalton
Kg	Kilogram
KNO_3	Potassium Nitrate
m	Meter
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization Time of Fight Mass Spectrometry
mg	Miligram
Mha	Million Hectares
ml	Mili Liters
MR-VP	Methyl-Red and Voges-Proskauer
N	Nitrogen
N_2	Dinitrogen
Na^+	Sodium Ion
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
NBAIM	National Bureau of Agriculturally Important Microorganisms
NH_3	Ammonia
NH_4Cl	Ammonium chloride
nm	Nanometer
OD	Optical Density
PBS	Phosphate Buffer Saline

PDA	Potato Dextrose Agar
PGP	Plant Growth Promoting
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-P-Hydroxybutyrate
PIPES	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
PSB	Phosphate Solubilizing Bacteria
PSI	Phosphate Solubilization Index
PSU	Percent Siderophore Unit
rpm	Rotation per Minutes
rRNA	Ribosomal Ribonucleic Acid
RWC	Relative Water Content
SD	Standard Deviation
SEM	Scanning Electron Microscopy
ST-PGPR	Salt-Tolerant Plant Growth Promoting Rhizobacteria
TCA	Trichloroacetic acid
UV	Ultra Violet
UV-Vis	Ultra Violet Visible
w/v	Weight by Volume
WHO	World Health Organization
XRD	X-Ray Diffraction
YEMA	Yeast Extract Mannitol Agar
Zn	Zinc
ZSI	Zinc Solubilization Index
α	Alpha
β	Beta
θ	Theta
ϒ	Gamma

INTRODUCTION

Ecosystem degradation and pollution has been a global concern with limited sustainable solutions. The injudicious anthropogenic activities have affected planet's nine habitability systems including land system change, climate change, biodiversity loss, biogeochemical cycling, freshwater use, aerosol loading, ozone depletion, novel entities and ocean acidification. Human interferences have worsened the scenario to such an extent that out of these nine systems, three (climate change, biodiversity loss, biogeochemical cycle) have already surpassed the permissible limits and land-system change is progressing in the risk-zone (**Fig. 1**; Steffen et al. 2015). The land system change is one of the major menaces that has highly affected our food basket and in the present situation 1 in 9 people are undernourished (FAO, IFAD, UNICEF, WFP and WHO 2021).

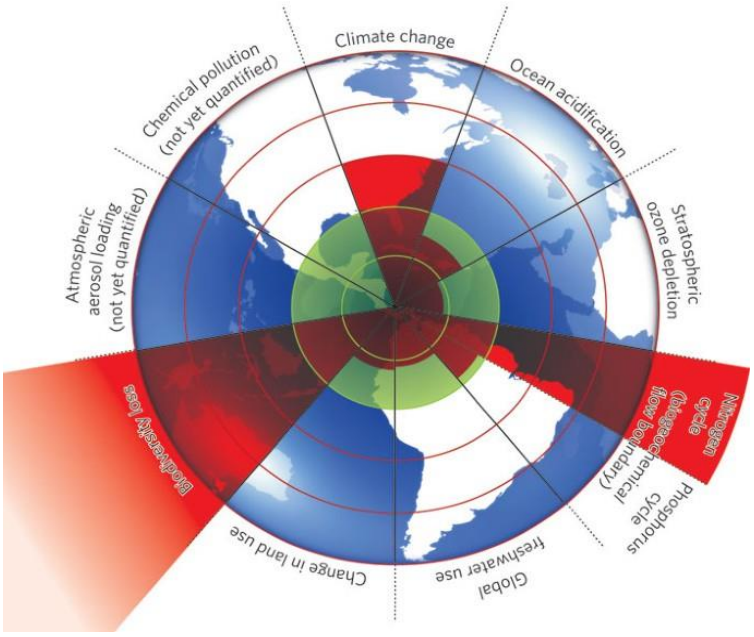


Fig. 1 Nine habitability systems of planet Earth ensuring the survival of life-forms.

The green shading represents the safe limit for the1 operation of nine habitability systems and red shading represents that the permissible limit for the variable has already

exceeded (for the three systems loss of biodiversity, nitrogen cycle and climate change) (Rockström et al. 2009).

The exponential increase in population is further depressing land availability and food production. Relatively, the projected population of 9.7 billion by 2050 (United Nation Population Division 2019) requires 70% increase in food requirements to feed the population in a sustainable manner (FAO 2011). The challenge in providing nourishment to population leads to increase in land degradation, compromising the quality and fertility of soil. The stats of last 14 years highlight that with 18.6% increase in human population, there has been only 2.6% increase in agricultural lands (FAO 2015). Categorizing the cause of lower agricultural land availability, UN Convention to Combat Desertification (UNCCD) declared land degradation as the major cause of reduction in soil health and productivity (UNCCD 2002). Globally, a quarter of land area is declared to be degraded resulting in annual economic loss of about US$ 230 billion annually (Lal et al. 2012; Nkonya et al. 2016). Land degradation leads to loss of 75 billion tonnes (Pg) soils from arable lands every year corresponding to financial loss of about US $400 billion (FAO-Global Soil Partnership 2017). Soil salinization and sodication is the second major cause (after soil erosion) of land degradation and creation of marginal areas across the globe, impacting the food security (Shahid et al. 2018a; Mukhopadhyay et al. 2021). Salt affected areas are mainly disturbed in arid and semi-arid areas due to their lower level of precipitation which resists the only solution of removing salts from the affected lands and this further exposes the salts to erosion and spreading across the neighboring lands. But gradually, the increased anthropogenic activities have led to salinization and sodication in almost 100 countries including the Polar Regions, irrespective of their climatic and demographic distribution (Buringh 1979; Shahid et al. 2018a; Hassani et al. 2020). Relatively, Orhan (2016) reported that

15% of the total saline soils are in arid and semi-arid areas while 40% are in irrigated lands. About 1 billion hectares of land are under salinity/ sodicity stress globally of which 351.2 million hectares (Mha) are saline and 581.0 Mha are sodic (Arora et al. 2018a; FAO 2020). Salinity leads to loss of 2000 ha of arable lands every day and 1-2% of fertile soil each year (Etesami and Beattie 2018; Arora et al. 2020a). About 23% and 37% of total cultivated land are under salinity and sodicity stress respectively (Shahid et al. 2018b). The recorded history of soil salinization provide evidence that civilizations like Mesopotamia flourished and then failed due to salinity. The publication '*Salt and silt in ancient Mesopotamian agriculture*' explains the historic record of human-induced salinization (Jacobson and Adams 1958). The major causes behind the episodes of salinization during ancient time were flooding, over-irrigation, seepage, silting, and a rising water-table (Gelburd 1985). Recently, evidences have been found that problems of salinity and sodicity prevailed in Indus plains of Pakistan and India 2000 years ago during Harapa civilization (Shahid et al. 2018a). Viru valley of Peru, also suffered relocation due to increased salinity, elevating water-table and poor soil drainage (Armillas 1961). Lately, during Green revolution there has been peaking figures of soil salinization (since 1945) and till date 17% of agricultural lands are lost due to human-induced soil salinity (Arora et al. 2018a).

Saline soils show presence of excess soluble salts including cations and anions: sodium (Na^{+}), calcium (Ca^{+2}), magnesium (Mg^{+2}), chloride (Cl^{-1}), carbonate (CO_3^{-2}), sulphate (SO_4^{-2}), nitrate (NO_3^{-1}); hyper saline soils even include boron (B), lithium (Li), strontium (Sr), selenium (Se), silica (Si), rubidium (Rb), barium (Ba), manganese (Mn), molybdenum (Mo), aluminium (Al), fluorine (Fl) leading to toxicity in various life forms (Tanji 1990). The defining property of saline soils according to glossary of saline soil in USA is the ECe (electrical conductivity) greater than 4 deci Siemens per meter

(dS/m) at 25ºC (Mishra et al. 2018). Saline soils can be visually recognized by their white crust. There are three major types of salt-affected soils: saline soil with >4 dS m^{-1} of ECe have an exchangeable sodium percentage (ESP) <15%, a pH below 8.5, and sodium adsorption ratio (SAR) of 0-12 typically identified by white incrustation; saline-sodic soil with ECe >4 dS m^{-1}, and a pH below 8.5, but have an ESP >15% and SAR >12; sodic soil containing few neutral soluble salts, the ESP is >15%, the ECe is <4 dS m^{-1}, and the pH above 8.5 (Scianna 2002; US Department of Agriculture, Natural Resources Conservation Service (USDA-NRCS) 2002; Doula and Sarris 2016). **Table 1** describes the classification of the salt-affected soils according to the ECe and pH aiding the interpretation of soil analysis results.

Table. 1 Classification of soils on the basis of salinity and its effect on crop growth (Data adapted from: Fan et al. 2012; Alonge et al. 2019)

Class of saline soil	**Electrical-conductivity of soil saturated extract (ECe) at 25º C (dS/ m)**	**Soil salinity in percent**	**Salt visibility**	**Effect of salinity on crop growth**
Non-saline	< 4	< 0.2	Salts not visible	Crop sensitivity
Slightly saline	4 - 8	0.2 ~ 0.4	Salts slightly visible	Crops can grow normally
Moderately saline	8 - 15	0.4 ~ 0.6	Salts fairly visible	Reduction of crop growth by about 50% to 60%
Strongly saline	> 15	> 0.6	Soil surface fluffy and salts fairly visible	Crops do not survive, only salt-tolerant pastures can survive and reduction of crop growth is more than 70% and 80%

The aforementioned problems of excess sodium, ECe and pH in salt-affected soils adversely impacts the physical properties of soil thereby leading to poor growth of

plants. Low permeability of soils leads to anaerobic conditions and upon drying the soil strata hardens inhibiting seed germination and root penetration. The high salt concentration increases soil osmotic pressure and thereby restricts the movement of nutrition, minerals and water into the plant (Machado and Serralheiro 2017). Limited water and carbon dioxide (CO_2) diffusion rate and reduced photosynthetic pigments in plants leads to lowered photosynthesis under salt stress. Stomatal closure, lower water retention, cell plasmolysis, cell membrane flaccidity, ethylene stress are other detrimental responses of salinity and sodicity in plants (Arora et al. 2016a, 2018a). Marcelis and Hooijdonk (1999) reported that 80% growth loss in radish under salt stress was due to reduced leaf area expansion leading to minimal light interception and the remaining 20% was because of inhibited stomatal conductance. The accumulation of toxic ions such as B, Se, Al, sodium, chloride (NaCl) inhibits protein synthesis, inactivates enzymes, modify the molecular mechanisms in plants, damages chloroplast and other organelles (Taiz and Zeiger 2002). Salinity also imposes osmotic imbalance and water stress, under such conditions the microbial cells lyse and dry out leading to reduced soil biomass. The decline in microbial status leads to disturbed carbon (C) and nitrogen (N) mineralization which are mainly biologically mediated (De León-Lorenzana et al. 2018; FAO 2019). Correspondingly, Arora et al. (2018b) reported that in saline regions of Kanpur, Uttar Pradesh, India the legumes showed poor nodulation, and 80% population of the surveyed pigeon pea plants were either poorly or non-nodulated due to salinity. Similar to the nodulous plants, sunflower is also negatively impacted by salinity, reducing the germination rate, nutrient and water uptake (Li et al. 2020; Bakhoum et al. 2020). Though sunflower is moderately salt tolerant yet increasing salinity compromises the growth and yield of crop. Being an important oilseed and cash crop, sunflower also holds both health and industrial benefits (Fatima

and Arora 2021). The elevation of salt-stress in plants through biological methods such as use of bioinoculants can help to increase the oil production , which would thereby benefit the farmers to uplift their standards of living as well as economy of nation. India is one of the major producer of oilseed crops in the world yet sunflower productivity has seen a sharp decline of 78% in productivity and 81% in area of production in the country in last 30 years; additionally Uttar Pradesh has lost its status of sunflower production in India since 2014-2015 (Ministry of Agriculture https://agriexchange.apeda.gov.in/About_Agri_Exchange.aspx). Therefore, targeting an important cash crop with moderate salt tolerance would help to regain the status of sunflower production in the state (UP) and also to dilute the challenges faced by the plant (i.e. salinity) nationally and globally, sustaining the food basket.

The treatment and reclamation of saline and sodic soils is still on spurious grounds and requires a suitable method for their rejuvenation. Salt affected soils can be remediated using physical, chemical, hydrological and biological methods. Different physical methods used are leveling- leaching of salts using uniform water distribution followed by plowing the fields; subsoiling- particularly important in treating sodic soils by dispersing underlying dense clay-sodic layers to increase water permeability; sanding- mixing sand with heavy textured soil; tillage- reshaping soil beds to reduce salinity effects; scraping- physically removing salt crust (Ezeaku 2011). Chemical methods of treatment include amendment of soil with gypsum, elemental sulphur and acids (Birru et al. 2019). Hydrological methods include reclamation using irrigation systems to leach and drain salts. These methods of reclamation require a high energy input along with high per capita investment and even the remediation strategies are either temporary or site specific and cannot be an effective approach in rejuvenating saline and sodic soils. Thus, the green biological approach of using beneficial microbes (term referred

as rhizoremediation) are regarded as the most sustainable solution with objective of permanent, cost effective and eco-friendly remediation.

Plant growth promoting rhizobacteria (PGPR) are rhizosphere residing microbes which show beneficial association with plants, promoting their growth and increasing nutrient strata and structure of rhizoplane under stressed and non-stressed conditions. The two-way mechanisms of reclamation by microbes involve growth promotion and stress tolerance in plants and improvement of the quality of soil through soil aggregation, nutrient chelation and hydration (Kumar et al. 2020). Various mechanisms involved in providing resilience to plants include production of exopolysaccharides (EPS), osmoprotectants/ compatible solutes (proline, glycine betaine, trehalose, sucrose), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD), restricting the uptake of Na^+ (sodium ions) by changing the composition of cell membrane along with increase in expression of salt-overly sensitive (SOS) genes and NHX transporters in plants (Pinedo et al. 2015; Egamberdieva et al. 2019; Fatima and Arora 2021). Apart from defense mechanisms, salt-tolerant PGPR also induce the growth of plants through phytohormone production indole-acetic acid (IAA), gibberellic acid (GA), cytokinins (CKs)), nutrient assimilation (N, phosphorous (P), potassium (K), iron (Fe), zinc (Zn) chelation) and improvement of soil structure (Shahzad et al. 2017). The consecutive application of microbes over the years improves the productivity of soil and supports the growth of plants, mitigating the ill-effects of salt. Lately, rhizoremediation technology has also shown increase in market value estimating about US$ 32.2 billion in 2016 and is expected to increase to US$ 65.7 billion by 2025 at a CAGR of 8.3% from 2017 to 2025 (Arora et al. 2018a).

The unbalancing of ions in plants under salt stress primarily requires maintenance of osmotic equilibrium through high cytosolic K concentrations and low Na accumulation in the cytoplasm and cytosol (Khan et al. 2021). Secondarily, the morphological changes due to salinity, affecting plants at various stages including germination, seedling, vegetative, and mature stages and biochemical changes modulating phytohormone and nutrient levels require adaption and resource using efficiency in the stressful environment. At molecular level salinity also challenges regulation of important genes associated with photosynthesis, hydration, hormonal balancing and various signaling networks and pathways in plants (Sunita et al. 2020). Elaborating role of PGPR in alleviating salt stress in plants, these beneficial microbes improve the morphological, biochemical and molecular processes in plants thereby rescuing yield and productivity loss. The amelioration strategy of salt tolerant PGPR (ST-PGPR) involves triggering of response systems in plants at the onset of stress followed by synthesis of anti-stress biochemical, enzymes and metabolites such as antioxidants, osmolytes, EPS, ACCD along with production of growth-regulators including phytohormones and organic acids (for nutrient uptake) (Fouda et al. 2019; Ha-Tran et al. 2021). Inspecting the mechanism of action, Arora et al. (2020a) illustrated that to avoid desiccation and flaccidity, ST-PGPR pool out the toxicity through changes in membrane structure, increasing electrogenic Na^+/H^+ ionic-porters, along with increased expression of *SOS* genes and NHX transporters in plants. Accumulation of low molecular weight osmolytes further lessen the ionic toxicity in cytoplasm and establish the equilibrium thereby maintaining the integrity and turgor pressure of cells (Fan et al. 2020; Gregory and Boyd 2021). Additionally, in order to restrict the uptake of Na^+ ions, microbes also produce EPS as a physical barrier and an active exchange site of nutrients and signals, cation uptake, symbiotic associations and nodulation (Ayangbenro and

Babalola 2021). Due to hygroscopic nature of the polysaccharides, these macromolecules also facilitate the hydraulic conductivity and aid in maintaining the water holding capacity of soil thereby improving aggregation and nutrient chelation (Sahu et al. 2019; Biswas et al. 2020). The slimy and capsular EPS also help in increasing the root colonization of rhizospheric microbes through the presence of fibrillar materials, hence increasing the microbial activity in arid hostile soils (Fatima and Arora 2021). Relatively, Fatima et al. (2020) elaborated the role of EPS in mitigating salt stress in rice plants when applied at various concentrations. The treatments improved the growth parameters of plants and EPS was found to be the most dominant response of *Alcaligenes* sp. AF7 strain under salinity. Gan et al. (2020) reported production of novel EPS from halophilic bacteria *Halomonas saliphila* LCB169T, exhibiting outstanding water solubility index, water holding capacity, oil holding capacity, emulsification activity and foaming capacity. Rasulov et al. (2020) predicted the metabolic and molecular pattern of EPS production by *Rhizobium radiobacter* SZ4S7S14. The study elaborated the changes in monomer composition upon increase in salinity and the biomolecule was also found to be an efficient Na^+ chelator.

Arthrobacter, Azospirillum, Alcaligenes, Bacillus, Burkholderia, Enterobactor, Flavobacterium, Pseudomonas and *Rhizobium* are some of the halotolerant soil bacteria evinced in promoting growth of plant under hostile environment (Arora et al. 2020a). Although there are various reports regarding the effectiveness of ST-PGPR in diluting the impact of salinity in plants yet the intriguing interactions between the plant and beneficial microbes and their relation with salinity still requires further research and the present study is an effort to unfold this dynamic relationship. Shahid et al. (2021) found that colonization of *Vigna radiata* by halotolerant strain of *Kosakonia sacchari* MSK1

elicited salt-tolerance responses in plants and improved the ionic equilibrium, ROS scavenging activity and status of stress-mitigating metabolites under saline conditions. Similarly, Zuluaga et al. (2021) showed that inoculation of salt-stressed tomato plants with *Pseudomonas* 16S showed improvement in plant biomass and mechanism reported was the accumulation of primary and secondary metabolites such as low molecular weight osmolytes (e.g., amino acids), as well as anthocyanins, hydroxycinnamic acids, coumarins and saponins as well as ROS scavenging antioxidants.

Rhizoremediation of polluted soils has shown large scale results and variety of wild and genetically modified microbes degrading complex, xenobiotic compounds, however bio-reclamation of saline soils is still in preliminary stage and requires intense field application. Therefore, the reclamation strategy of microbes and loopholes in the technology are required to be re-designed to achieve a better future in overcoming the problems of soil salinity.

Objectives of the study

1. Isolation and selection of salt tolerant fluorescent pseudomonads from saline soil
2. Molecular characterization of the isolated fluorescent pseudomonads
3. Checking PGP traits of isolated fluorescent pseudomonads (production of IAA, HCN, ACC deaminase, EPS, siderophore and phosphate solubilization)
4. Isolation, purification and characterization of the metabolites involved in salt tolerance
5. Plant-fluorescent pseudomonads interactions under saline conditions in pot and field trials and checking the mechanism of fluorescent pseudomonads on the production of *H. annuus* in saline conditions
6. Checking the impact on yield, oil content and growth parameters of *H. annuus* under different saline conditions

REVIEW OF LITERATURE

Injudicious anthropogenic activities have generated a surpassing level of contaminated soils along with the salinization of lands, hitting hard the agro-ecosystems. About 70% of the countries suffer from various form of land-salinity problems (FAO 2020). Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES 2018) estimate that about 190 million acres of lands are completely lost; 150 million acres are damaged and 2.5 billion acres (around 1 billion hectares) are impacted by salinity. The global distribution of salinity highlights that there are around 21 countries with more than 15% of their land degraded under salt stress. Of these 21 countries, 13 are in a border belt extending from African Sahara, covering Sahel zone through Middle East and up to Central Asia. Similarly, six countries (of which three are in Central Asia) show strong levels of sodicity with more than 10% of their total land area under the stress (FAO 2000). At continental level, the highest saline areas are in Asia (including Middle East) with 7.14 Mkm^2 (Million km square.) of affected areas, followed by Africa with 2.292 Mkm^2, Australia and Oceania with 1.313 Mkm^2, South America with 0.527 Mkm^2, North America with 0.422 Mkm^2 and Europe with 0.024 Mkm^2 (**Fig. 2**). Countries with major salinity and sodicity constrains are China with 211.74 Mha, Australia with 131.40 Mha, Kazakhstan with 93.31 Mha, and Iran with 88.33 Mha (Hassani et al. 2020). The drying of Aral Sea with currently only one-tenth of its original volume being left, has eroded the neighboring areas of Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan (Arora et al. 2018a; Tussupova et al. 2020). The exposed lakebed is now eroded by wind, settling the salts in neighboring farmlands degrading the soil (https://earthobservatory.nasa.gov/world-of-change/aral_sea.php). Uzbekistan and Turkmenistan (fed by diverted Aral Sea) are degraded with 24% and 32% of their total land under salinity/ sodicity stress respectively (Qadir et al. 2008). The second most salt degraded countries are Djibouti

(in sub-Saharan Africa) and Paraguay (in South America) with 44% of their total land deteriorated by salinity/ sodicity. Mapping the countries with high levels of secondary (human-induced) salinization, most of them are located in arid and semi-arid regions including Chile, Argentina, Commonwealth of Independent States, Egypt, Iraq, India, Iran, Spain, Thailand, Pakistan, Turkey, Algeria, Syria, Sudan, Tunisia, Gulf States and China (Shahid et al. 2018b; Xu et al. 2019). The other highly salt-degraded regions of the world are- the Indo-Gangetic basin in India, the Indus basin in Pakistan, the Yellow River Basin in China, the Euphrates basin in Syria and Iraq, the Murray-Darling basin in Australia, the San Joaquin valley in the United States (Cherlet et al. 2018; Kumar and Sharma 2020).

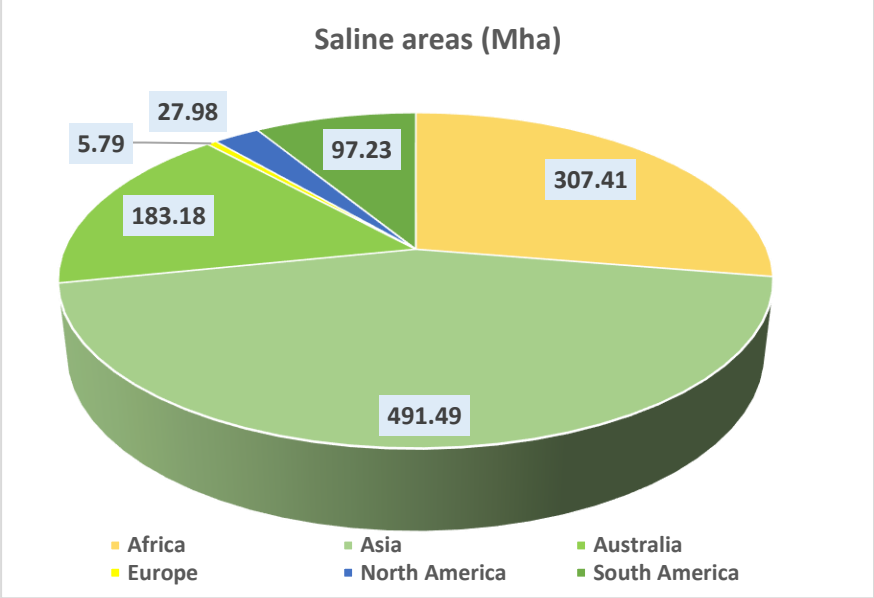


Fig. 2 Distribution of salt-affected lands in various continents across the globe between 1980-2018 (Tussupova et al. 2020)

The evidential changes in agricultural production, food security, crop losses, desertification due to increased anthropogenic activities clearly indicates that the

present scenario of salinization is more depressing and if actions are not taken nearly 50% of global land would be under the menace of salinity (Munns and Tester 2008).

2.1 Effect of salinity on physiology and growth of plants

Salinity leads to drastic loss in plant growth and productivity due to various physiological, developmental and biochemical changes. Rajabi Dehnavi et al. (2020) report that salinity is responsible for 98% of the variations seen in the plant parameters such as germination, root-shoot length of seedlings, seedling vigor index, seedling biomasss and salt tolerance indices. The two-phased plant response (**Fig. 3**) to salinity includes: primarily, within few minutes or days to salt-exposure, the stomata closes and cell expansion is inhibited mainly in shoots (Munns and Termaat 1986; Munns et al. 1995; Rajendran et al. 2009). The symptoms include yellowing of leaves, wilting and stunted growth. In the secondary phase after days or weeks the cytotoxicity of ions build-up and affects the metabolic processes, causes premature senescence and ultimately cell death (Munns and Tester 2008; Isayenkov and Maathuis 2019). The second phase symptoms include chlorosis of green leaves, leaf tip burning, necrosis and scorching (Shannon et al. 1998).

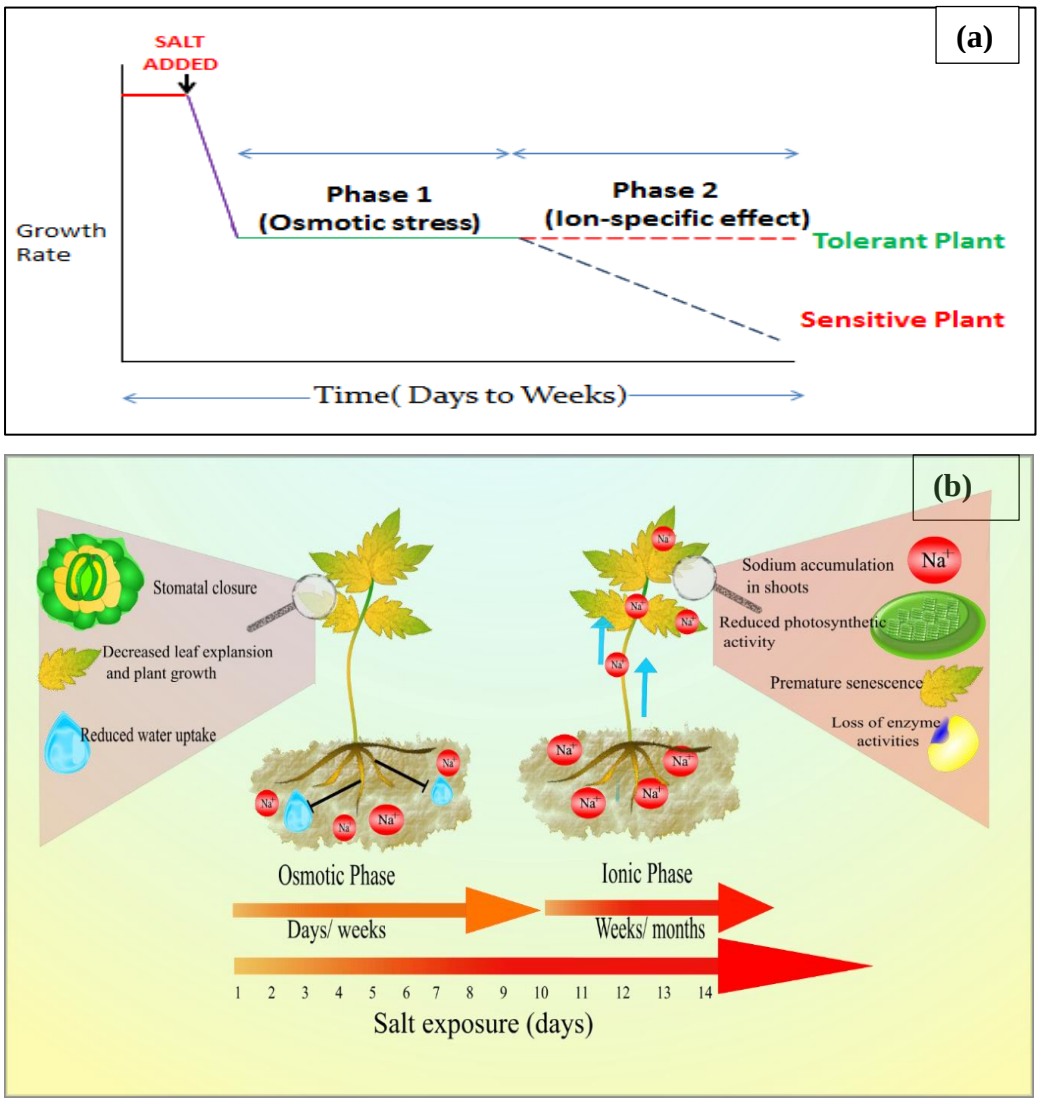


Fig. 3 (a) Schematic representation of two-phased plant response to salinity in both salt-sensitive and salt-tolerant plants and its effect on plant growth rate over time (Adapted from: Munns et al. 1995) **(b)** responses of plants to salinity and its effect on various physiological activities

The accumulation of salts in the root zone causes osmotic imbalance and disrupts ion homeostasis in cells, thereby inhibiting the uptake of essential nutrients such as calcium (Ca^{+2}), K^{+}, NO_3^{-1} (nitrate). The entrance of toxic salts (like NaCl) in the plant cell sap *via* symplastic and apoplastic pathway injures the chloroplasts and other organelles and also inhibits the photosynthetic rate, protein synthesis and leads to inactivation of enzymes (Taiz and Zeiger 2002; Maathuis et al. 2014; Ha-Tran et al. 2021). Salinity reduces the overall plant biomass, however strangely root biomass is generally found

to be lesser effected than shoot due to reasons unknown. Presence of excess sodium also affects the structure and fertility of soil. Generally, in normal soils organic matter, exchangeable cations such as Ca^{2+} and Mg^{2+} link the clay particles together to humic acids forming micro and macro-aggregates which enhances the porosity, water-holding capacity and internal drainage of soil (Machado and Serralheiro 2017). But saline soils with high concentration of sodium, replaces calcium with sodium and due to lower flocculation ability of the latter, dispersion of soil particles take place. Therefore, the presence of high levels of exchangeable sodium leads to poor plant growth and even creation of marginal lands. Relatively, Aydinşakir et al. (2015) reported more than 20% loss in root and shoot biomass of *Arachis hypogea* when the salinity of irrigation-water increased to 4 dS/m. Khan et al. (2019) observed significant fresh and dry weight reduction in soybean plants with increase in salinity concentration from 100 mM to 300 mM NaCl. Exposure of alfalfa seeds to130 mM NaCl reduced the germination rate to 29% with severe effect on plant height, biomass and cell integrity (Zhu et al. 2020). Pushpavalli et al. (2016) reported high sodium toxicity in leaves leading to delayed flowering in *Cicer arietinum.* Illustrating the effect of NaCl-induced salinity, Alam et al. (2016) found significant impact on plant height, number of leaves and flowers, fresh and dry weights of purslane plants when the salinity elevated to 32 dS/ m. In plants like grapevine, citrus and soybean, it has been found that very low amount of Na^{+} reach the leaves, but the highest toxicity in plants is due to Cl^{-} (chloride) (Munns and Tester 2008; Shan 2009). Cao et al. (2015) showed that upon exposure of *Suaeda aralocaspica* to salinity there was drastic lowering of photosynthetic pigments and this was mainly due to destruction of chloroplasts. Photosystem II (PS II) is the most important pigment system involved in photosynthesis, correlatively, due to salinity, structure and activity of PS II gets highly disturbed (Saleem et al. 2011; Okon 2019). Explaining the effect

of osmotic unbalancing under salinity. Mumtaz et al. (2013) discussed enhancement of electrolyte leakage in *Cucumis sativus* plants as compared to non-salinized control plants. Water stress arising due to salinity leads to stomatal closure in order to prevent dehydration. However, these physiological changes and closure of stomata leads to lowering of transpiration and hence the temperature of plant gradually increases causing reduced photosynthetic rate and impaired growth (Jose et al. 2017). Achten et al. (2010) examined that the biomass of well-watered *Jatropha* plants was 57% more than water stressed plants. Estimating the drought and salinity responses in moderately tolerant *Jatropha curcas,* Abrar et al. (2020) revealed that stressed plants had almost 93% more Na^{+} content in leaves, and lowest membrane stability index (MSI) and relative water content (RWC) as compared to control unstressed plants. The disturbed K^{+1}: Na^{+1} ratio was the major cause in limiting the growth and development of plants. Defining the influence of salt-toxicity on nitrogen fixing ability of plants, Shao et al. (2019) explained that although *Elaeagnus commutate* seedling supported growth and tolerance up to 100 mM NaCl condition, yet there was about 98% loss in nitrogen fixing ability of plants as compared to unstressed control. Relatively, Abd-Alla et al. (2014) observed 43% and 100% reduction in nodule number formed by infection of *Rhizobium tibeticum* in fenugreek under 100 and 150 mM NaCl conditions respectively. Further, the study also reported 53 and 100% reduction in nodule fresh weight under 100 and 150 mM NaCl respectively.

2.1.1 Effect of salinity on oilseed crops

Oilseed crops are the most important agricultural commodities due to their economic value. The increasing demand of oilseed crops for healthy vegetable oils, livestock feeds, pharmaceuticals, biofuels, painting, emulsion, wax, oleo-chemical and other industries has led to 82% increase in oilseed crop cultivation and 240% increase in

world production over 30 years (1983-2013) (Rahman and de Jiménez 2016). However, irrespective of the overall increase in production, the trend is different for individual oilseed crops with both increment as well as decrement in their productivity. Five major oil crops are: oil palm (*Elaeis guineensis*), soybean (*Glycine max*), oilseed rape (*Brassica napus*), sunflower (*Helianthus annuus*), and olives (*Olea europaea*) accounting for 80% of world oil production (FAOSTAT 2014). The distribution and cultivation of these crops are popular both in developing and developed countries and the variation in demographic, temporal and geographic location determines the impact of climate change on each crops. Though the production of oil-seed crops has increased over the time span, yet the sharp increase in demand is somehow unbalancing the availability per person. Additionally, climate change is expected to put pressure on oilseed crop performance and productivity. The expected increase of 1.6 to 6^{o}C in temperature by 2050 along with abnormal precipitation pattern across the globe would challenge the races and wild relatives of oilseed crops (IPCC 2013). For example, it is presumed that by next 50 years about 61% of *Arachis* spp. (peanuts; an oilseed crop) would be extinct due to climate change (Jaradat 2016). Jaime et al. (2018) report that increase in temperature and aridity in many temperate regions impact the production of oilseed crops and resultantly, development of new alternative crops adapted to more xeric conditions is the new approach in Mediterranean climate regions. However, this approach is cost-ineffective and energy and time consuming and cannot be adapted for different demographic regions. Sharif et al. (2017) found that intense rainfall during autumn and winter causes incidence of disease and yield loss in winter oilseed rape. Due to sea level rise, flooding of lands with seawater causes salty soils. This seawater flooding causes reduction in biomass and productivity of oilseed crops due to lower siliques per plant and seed mass (Hanley et al. 2019).

Fahad et al. (2017) elaborate that heat stress causes significant reduction in oil and protein content of seeds. Beillouin et al. (2020) also predicted through machine learning models that severe heatwaves and frequent droughts in Europe in the year 2018 caused about 65% of historical yield anomalies including significant loss in production of oilseed rape. Specifying about the effect of salinity on oilseed plants the response varies according to the type of crop, soil properties, genotypes and developmental stages. Most of the oilseed plants are sensitive to salinity and depending upon their tolerance and sensitivity levels crops like canola are regarded as salt-tolerant withstanding EC upto 10 dS/ m while linseed regarded as salt-sensitive showing growth loss even at EC 2 dS/ m or above (Nayidu et al. 2013). Naveed et al. (2020) reported that salt-toxicity in canola caused significant loss in growth parameters and physiological traits such as reduced photosynthetic rate, stomatal conductance, transpiration rate, chlorophyll content, intrinsic CO_2 level, RWC and enhanced electrolyte leakage, ROS generation and Na^+ level in leaves. Based on 2014-15 estimates elaborate that due to salinity, India loses 16.84 million tons of cereals, oilseeds, cash crops and pulses production annually accounting to 230.20 billion INR (Indian Rupees) (Mandal et al. 2018; Kumar and Sharma 2020). Abdel Latef et al. (2021) also reported significant growth loss of canola plants grown in saline soil due to enhanced oxidative and water stress.

Ranking the global market of oilseed crops, soybean is the largest produced (60%), followed by rapeseed (12%) and sunflower (9%) (Pilorgé 2020). Estimating the worldwide oilseed production in 2020/ 2021, soybean production was reported to be 362.05 million metric tons, 68.87 million metric tons for rapeseed and 49.46 million metric tons for sunflower (Statista 2021; **Fig. 4**).

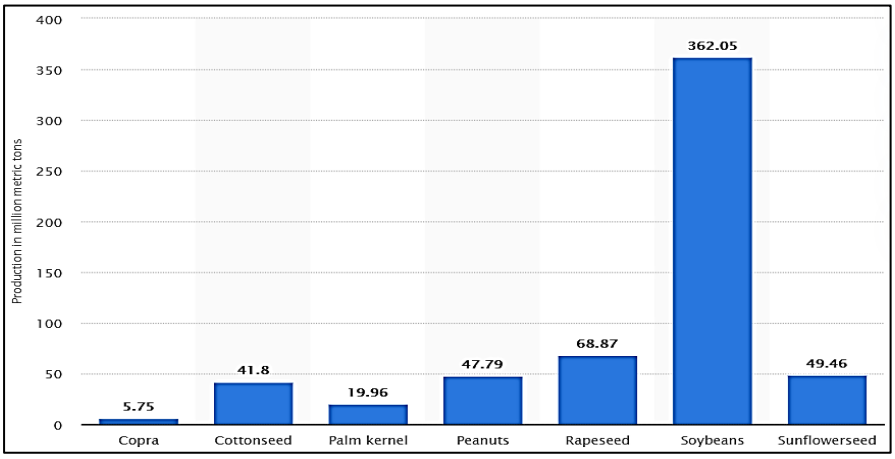


Fig. 4 Oilseed production of different crops worldwide in 2020/21 (in million metric tons) (Statista 2021)

Sunflower, an Asteraceae (Compositeae) family plant, is produced on large scale in only few countries and the top ten are Ukraine, Federation of Russia, Argentina, China, Romania, Bulgaria, Turkey, Hungary, France and USA (in order of their productivity from period 2009 to 2018) (Oil World Annual 2019; **Fig. 5**).

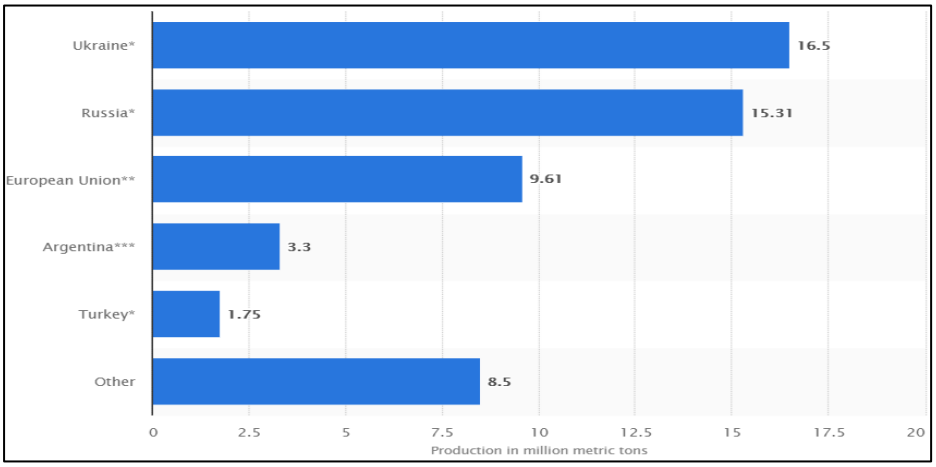


Fig. 5 Production of sunflower in various countries across the globe (in million metric tons) in 2019/20 (Statista 2021)

The seed of sunflower contains 44% oil and 16% protein, therefore, it competes on both vegetable oil and protein market (Pilorgé 2020). The high nutritional value of sunflower oil due to the presence of unsaturated fatty acids (oleic, linoleic, linolenic) and lower saturated fatty acids (palmitic and stearic) and absence of trans fats makes it superior to other vegetable oils (Premnath et al. 2016). Sunflower seed is also used as animal feed, snack, salad garnish, and in some bakery goods. The presence of phenolic compounds, flavonoids, vitamins, antioxidants, anti-inflammatory, anti-hypertensive properties in seeds aid in wound healing (Fowler 2006). Production of emulsions and margarine formulations, lubricants, biodiesel, vegetable-oil based inks; some by-products such as hulls, soapstocks, lecithins, tocopherols, waxes and meals are other industrial non-edible uses of sunflower (Grompone 2005; Salas et al. 2015). Pollution and climate change has modified the agro-systems and has led to changes in growth and yield of sunflower. As explained earlier, although there has been increase in overall oilseed crop production, but for sunflower there has been about 10% reduction in sunflower oil yield from the year 2019/2020 to 2020/2021 (USDA 2021). In India alone, from the year 2016 to 2020 there has been about 47 % decrease in sunflower yield with a lack of about 43% against the targeted production and 46% lacking against targeted cultivation area (Statista 2021; Directorate of Economics And Statistics (DES), 4th estimate (2019-2020); https://archive.pib.gov.in/documents/rlink/2019/aug/p201981901.pdf; **Fig. 6**). These depressing figures have already shifted the rank of India from fifth to ninth in worldwide production and the scenario is worsening each day (http://www.commoditiescontrol.com).

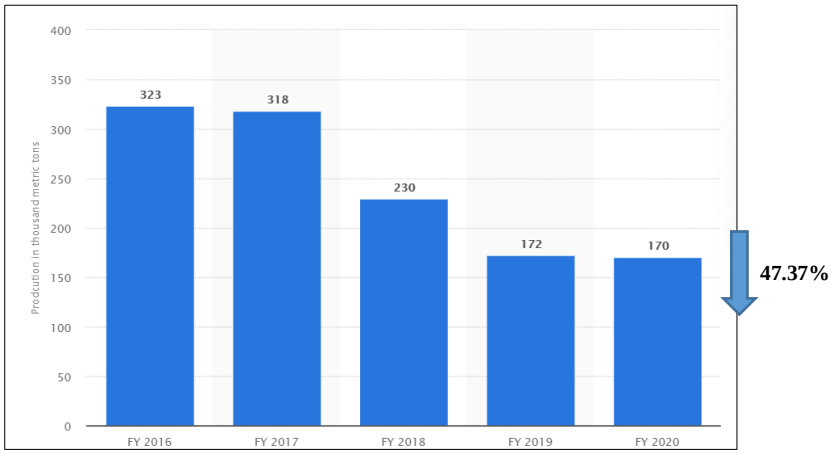


Fig. 6 Production of sunflower in India and the decline in productivity reported during 2016-2020 (Statista 2021)

Among the abiotic stresses, salinity has declined the productivity of the crop in many countries. Although sunflower is a moderately salt-tolerant crop but ECe beyond 2.5 dS/ m causes more than 30% yield losses (Khatoon et al. 2000; Li et al. 2020). Salt stress effects the growth and physiology of sunflower plant both at vegetative and reproductive stages. The salt toxicity mainly impacts the crop's oil content and in addition leads to delaying of flowering and maturation processes, reduces the production of glycerides and modification of fatty acids (determinants of oil quality) along with impaired leaf health, root/ shoot length and oxidative damage (Temme et al. 2019; Sunita et al. 2020). Thus, with the lacking of large scale remediation techniques, there is an urgent need to apply biological measures and compensate for the losses in a sustainable and cost-effective manner and fulfil the demand of increasing population.

2.2 Plant growth promoting rhizobacteria and the diversity of salt-tolerant PGPR across the globe

Microbes are natural integrity of soil systems, participating and affecting the cellular and metabolic cascade of events in the life cycle of a plant or the biogeochemical cycle

and nutrient content in soil. Further narrowing the zone of microbial proliferation, the small volume of soil enriched physically, chemically and biologically with plant roots and their signals referred as 'rhizosphere' by Hiltner (Hiltner 1904) are the hotspots of microbial activity. This zone is rich in nutrients, root exudates such as organic carbon compounds, amino acids, signalling molecules generally called as rhizodeposition (McNear 2013). These depositions make the rhizosphere a desirable niche for microorganisms and therefore, this area holds 10-100 times more microbes than in the remaining bulk of soil (Weller and Thomashow 1994). With diverse population of microbes in the rhizosphere only 1 to 2% are plant growth promoters (Antoun and Kloepper 2001). Although, the presence of beneficial nitrogen fixers housing inside the leguminous plants i.e., rhizobia, were discovered back in 1888 but the vast spectra of other plant growth promoters were still hidden and untold to the world. It was in 1978, when the agricultural world was focusing on microbial biocontrol agents, Kloepper and Schroth (1978) in their research found that some bacteria residing in the rhizosphere had no antagonistic activity yet they were tremendous plant growth promoters. This "unintentional" discovery marked the onset of new technology in agro-microbiology field and the researchers (Kloepper and Schroth 1978) credited the term "plant growth promoting rhizobacteria" to these significant entities of soil. Today several researches have reported the role of PGPR in increasing plant growth and yield and various PGPR have been licensed for use biofertilizers and bioinoculants (Arora et al. 2020b; Basu et al. 2021). The popularity of microbial soil inoculants has been gaining attention with market value of USD 396.07 million in 2018 at annual growth rate of 9.5% to reach the projection of USD 623.51 million by 2023 (Market Data Forecast 2018).

With the advancement of research, PGPR have also found their role in mitigating hostile conditions. Being the first habitants of Earth, microbes have tremendous

adaptive and tolerating properties aiding their survival in challenging conditions such as salinity. The traditional approaches to estimate the microbial biomass under stressed conditions elucidate a correlative loss in microbial community. However, with the advancement of technologies, microcosm experiments and next generation sequencing (NGS) have detailed the picture of microbial survival in inhospitable conditions. Detailing the diversity spectrum, modern systems and omics illustrate that there is pool of different microbial phylotypes and a shift in microbial community on exposure to salinity or other stresses (Zhang et al. 2019a). This concept of phase shift and adaptation broadens the knowledge about microbial ecology and conclude that microbial communities are regulated by both deterministic and stochastic processes. Linearly, deterministic refers to biotic interactions such as parasitism, mutualism; and stochastic processes refer to change in demography leading to mortality or passive stress (Caruso et al. 2011; Stomeo et al. 2013; Evans et al. 2017).

There is a rich diversity of salt tolerant PGPR reported in various countries by many researchers, explaining their role in improving plant growth and soil structure. Researchers have found heterogeneity in microbial behavior and tolerance capacity distinguishing their characterization as halophilic, alkaliphilic and also many novel salt tolerant microbial taxa or strains. According to Zhang et al. (2019a), salinity is a limiting factor defining the dissimilarity between the microbes. The study concluded that more the difference of salinity and distance between two sites more is the dissimilarity in the microbes. The dominant microbial phyla isolated by the workers were *Actinobacteria, Proteobacteria, Bacteroidetes, Chloroflexi* and *Firmicutes*. A catchy concept of the study was that observing the microbial diversity of Gurbantunggut Desert, Northwestern China, the authors found 22.5% and 18.2% variation in number of operational taxonomic units (OTUs) (indicating closely related

groups) and Faith's phylogenetic diversity (indicating dissimilar groups) respectively. Thereby, specifying phylogenetic diversity of microbes due to salinity responses. Keshri et al. (2013) also found the dominance of *Actinobacteria, Firmicutes, Proteobacteria* and archaeal taxa *Halobacteraceae* in saline-sodic soils, the library illustrated presence of 20% novel bacteria, 38% archaeal bacteria with ≤ 95% similarity to identified sequence indicating novel species. Shurigin et al. (2019) indicated presence of *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* along with *Alphaproteobacteria* and *Gammaproteobacteria* affiliated genera like *Roseovarius, Idiomarina* and *Spiribacter.* These genera and groups have been reported as efficient PGPR and their performances have been established under salinity stress. The most commonly reported salt-tolerant bacteria (with tolerance level 1-15% NaCl) are *Ochrobactrum intermedium, Bacillus subtilis, Pseudomonas fluorescence, Kocuria rhizophila, consortium, Bacillus amyloliquefaciens, Bacillus firmus, Azotobacter chrooccocum, Pseudomonas stutzeri, Azospirillum brasiliense, Azospirillium lipoferum, Pseudomonas putida, Curtobacterium flaccumfaciens, Arthrobacter sp.,* and *Bacillus safensis* (Shilev 2020). Misra et al. (2017) isolated salt tolerant *Bacillus spp.* from the saline regions of Indo-Gangetic plains with ACCD activity and other plant growth promoting (PGP) properties such as indole acetic acid (IAA), siderophore production, phosphate solubilization. Damodaran et al. (2013) reported salt tolerant strains of *Bacillus pumilus* and *B. subtilis* isolated from sodic regions of Indo-Gangetic Basin showing PGP traits including IAA, ammonia and hydrogen cyanide (HCN) production, phosphate solubilization even under salt conditions. The location of very first human civilization and also historically evident primary hotspot of secondary salinization is Indus-basin in Pakistan. The basin is now one of the most salt degraded lands in the world. Thriving in the vicinity of such hyper-osmotic environment, isolated

PGPR survive and show reclamation strategies promoting vegetation in the area. Correspondingly, Akram et al. (2016) concluded that isolate *Staphylococcus sciuri* isolated from Punjab, Pakistan showing sodicity tolerance and PGP properties incrementing the growth of maize under stressed conditions. Similarly, Naz et al. (2009) isolated salt-sodic tolerant PGPR from the rhizosphere of different weeds in the Khewra salt mine, Pakistan, which is the world's second largest salt mine. Zhu et al. (2011) reported *Kushneria sp*. YCWA18, a halotolerant (capable of growing upto 20% NaCl conditions) phosphate solubilizing strain isolated from the sediment of Daqiao saltern on the coast of Yellow Sea in China, which is one of the most saline areas of the world. The rich spectrum of salt tolerant microbes found across the globe even in extreme unhabituated environment initiates the hope of reclaiming saline and sodic soils.

2.3 ST-PGPR and their reclamation strategies

The biological approach of using salt-tolerant PGPR (ST-PGPR) to recover saline and sodic soils has received considerable recognition throughout the globe. The microbial strategy of salt tolerance is termed as "Induced Systemic Tolerance" by Yang et al. (2009) summarizing the chronological responses of microbes to insulate against the toxicity of salts. The survival of ST-PGPR in saline environment involves following hypotheses (i) limiting the uptake of salts due to cell membrane composition, (ii) extricating toxic ions of the cells through Na^+/H^+ antiporters and K^+ /Na^+ ion transporters adjusting osmotic conditions, (iii) endogenous biosynthesis of compatible solutes equalizing ionic status (iv) chaperons, proteins and enzymes to guide proper folding of protein and maintain the metabolic cascades (v) EPS production to form hydrated biofilm and improve the soil structure (Qin et al. 2016; Etesami and Beattie 2018; Arora et al. 2020b). **Fig. 7** illustrates the comparative analysis of salinity effects on plants and the action of ST-PGPR in combating the stress.

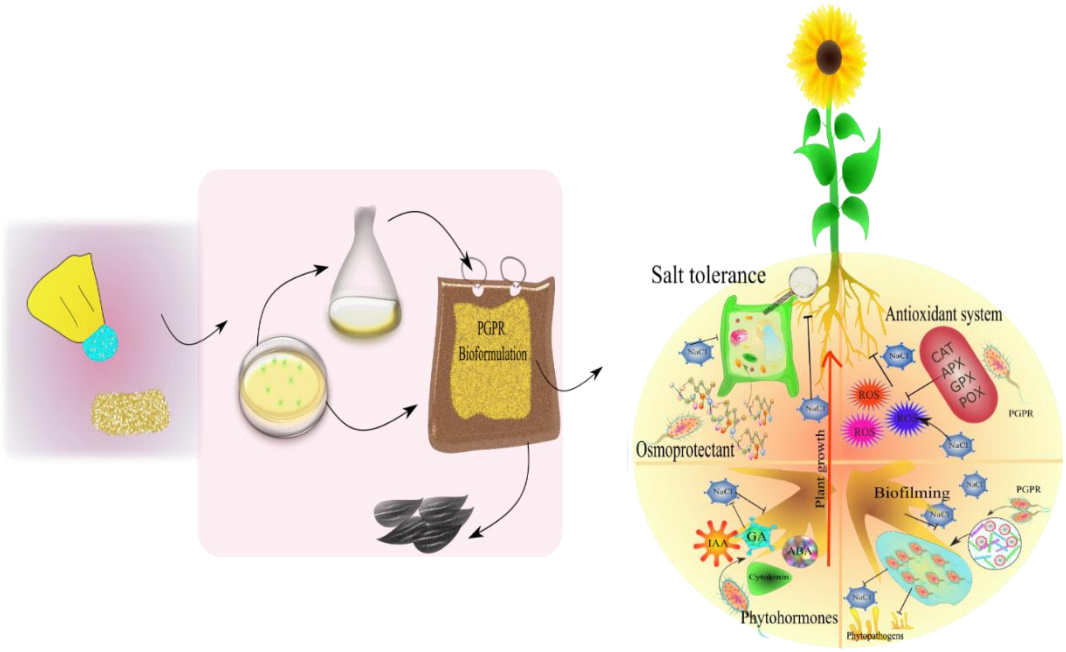


Fig. 7 Diagrammatic representation of salinity effects on plant growth and physiological properties versus the mechanism of action of salt-tolerant PGPR inducing tolerance responses and stress mitigation in plants

Cohering with the survival approaches, the application of PGPR reclaims the saline-sodic soils through two-way processes: firstly, promoting plant growth to increase the productivity and decrease the creation of marginal lands; secondly, to improve the quality of soil by ameliorating the structure, mineral and hydration status of infertile salty lands preventing them from erosion and water logging. The detailed role and mechanism of action of ST-PGPR are mentioned in the following sections.

2.3.1 Role of ST-PGPR in improving quality and structure of salt stressed soils

Soil fertility is determined by various factors including carbon content, hydration, water-holding capacity, NPK levels and the physical properties (Gouda et al. 2018). The holobiont beneficial association between microbes, plants and soil hold the co-evolution and capability to improve the soil fecundity, economic equality and feeding the population today and in future. The direct and indirect mechanisms of PGPR play

an important role in revitalizing the saline-sodic marginal lands. The toxicity due to Na^{+}, Cl^{-1} is the major menace diluting the productivity of soils. PGPR mitigate the toxicity of ions through cation bridging, hydrogen bonding, Van der Waals forces and anion adsorption thereby reducing their availability in soil and lowering the pH and ECe (Sandhya and Ali 2015). Correspondingly, Arora et al. (2016b) showed that the halophilic strains *Planococcus maritimus* CSSRO2 and *Nesterenkonia alba* CSSRY1 removed sodium ions from the soils. PGPR produce natural polymers like EPS which play major role in adsorption of toxic ions. Benefitting through their gummy nature and presence of charged moieties including hydroxyl, sulfhydryl, carboxyl and phosphoryl functional groups, EPS attract ions from the surrounding; balancing the ions levels (Fatima et al. 2020). Another challenge of soil salinity and toxicity is the hydration property. The production of EPS by microbes alters the structure of saline-sodic soils by forming aggregates and further sheathing the aggregates in a hydrated environment. Tisdall and Oades (1982) reported a sort of hierarchical model behind aggregate formation. Elucidating the model, the larger aggregates are composed of smaller units which further comprise microaggregates. In these micro-aggregates (2-20 µm diameter) the clay particles are adhered through binding agents such as soil polysaccharides, aluminosilicates, oxides, humic substance. The second strata of aggregation involves microaggregates clinging together to form larger microaggregates (20-250 µm diameter) through plant roots and root hair. Finally, larger microaggregates are glued together to form macroaggregates (> 250 µm diameter) which is again aided by polysaccharides and polyuronides (Costa et al. 2018). These aggregated systems not only reduce soil erodibility but also consist of internal complex pore systems for the exchange of water, gases and transport of dissolved compounds and colloidal particles and to harbor microbial communities (Totsche et al. 2018). The three dimensional

aggregate structure comprising soil and microbes has been conceptualized as 'self-organizing' by Young and Crawford (2004) and any change in the architecture can have major implications on hydration cycles, biological activities, and biogeochemical cycles of carbon, nitrogen and other elements (Totsche et al. 2018). Qurashi and Sabri (2012) found that the isolated strains *Planococcus rifietoensis* (RT4) caused maximum 808% increase in soil aggregation followed by *Halomonas variabilis* (HT1) with 666 % increase at 100mM NaCl condition.

The remediation and reclamation of soil has majorly been focused on intracellular microbial contents and the associated mechanisms, however, it is surprising that 99% of microbes on Earth survive in biofilms under diverse environmental conditions (Wu et al. 2019). EPS accounting for 80% of soil biofilm's dry mass (Chenu 1993) is *de facto* the microbial survival and soil architectural molecule. The high carbon (C) content in EPS is attributed as the important factor in coagulating the soil particles. This high C value in EPS also participates in C biogeochemical cycle and regulates C level which otherwise is highly reduced in saline-sodic soils. Bashan et al. (2004) confirmed aggregation of soil by EPS producing *Azospirillum* strains. Fatima and Arora (2021) elaborated the role of EPS in soil-aggregation and concluded that EPS produced under salinity (2% NaCl) showed better flocculation activity (of saline soil) as compared to non-salinized EPS. Similarly, Aureen and Saroj (2009) reported sand aggregation by *Microbacterium arborescens* AGSB. Correlatively, Shultana et al. (2020) reported that EPS from *Bacillus aryabhattai* UPMRE6 induced flocculation and aggregation along with adsorption of Na^+, thereby alleviating salinity stress and promoting growth of rice.

Saline soils are also characterized by low N content, due to decreased microbial activity especially of free-living or symbiotic N fixers. Lugtenberg et al. (2002) reported that

about 65% N supplied to plants are by soil microbes including rhizobial group and AMF, of which rhizobial-N-fixation contributes to about 20 to 40 Tg N per year (Herridge et al. 2008; Galloway et al. 2008). The integrative system of N fixers increase the mineral content of soil by fixing atmospheric nitrogen to ammonia. Kuan et al. (2016) reported that *B. subtilis* UPMB10 PGPR strain increased the nitrogen fixing capacity to about 30.5% and 25.5% after 50 and 65 days of treatment respectively. Similarly, Park et al. (2017) confirmed that *Bacillus licheniformis* MH48 increased soil nutrient *through* nitrogen fixation. Arif et al. (2017) explained that the enrichment of saline sodic soil with PGPR along with N enriched compost improved the N content of soil and also the growth of test crop (sunflower) after consecutive application for 2 years (2014-2015). Hassan and Bano (2019) concluded that *Pseudomonas moraviensis* inoculation ameliorated the saline-sodic soil by- increasing the P, NO_3^-, N and K contents by 18 – 35%. Orhan (2016) determined that the application of *Bacillus* strains OUT-142 and M3 significantly increased the fixed nitrogen in soil.

P is another limiting macronutrient in saline soils. Although P is abundant in soil yet the availability of soluble-form is deficient. Microbes play a significant role in regulating natural biogeochemical cycle of P. Since 1960s the tradition solution of applying NPK chemical fertilizers is effective but unsustainable, bruising the land by further increasing salinity. Thus, the only alternative is biological tools including the chelation of P through phosphate solubilizing microbes (PSM). The production of low molecular weight organic acids (succinic, gluconic, malic, oxalic, acetic, isobutyric, citric, maleic, lactic, aspartic), inorganic acids (sulphuric, nitric, and carbonic acids) phosphatase enzymes and ion exchange supports the assimilation of P from insoluble complexes in soil (Etesami 2018; Egamberdieva et al. 2019). Relevantly, Park et al. (2017) reported increase in P content of saline soil by inoculation with *B. licheniformis*

MH48 via organic acid exudation. Zhu et al. (2011) reported *Kushneria* sp. YCWA18 as potent inorganic and organo-phosphorus solubilizer along with its halophilic property. Strains such as *Aerococcus* sp. PSBCRG1-1, *Pseudomonas aeruginosa* PSBI3-1, *Aspergillus terreus* PSFCRG2-1 and *Aspergillus* sp. PSFNRH-2 are summarized as PSM by Srinivasan et al. (2012). Similarly, Jiang et al. (2018) characterized potent phosphate solubilizing halotolerant strain of *Bacillus* and *Providencia rettgeri* with high tolerance upto 1.5M NaCl, classifying them as efficient bioinoculants for remediation of saline soil. Jiang et al. (2020) also showed diversity of PSM including *Pseudomonas*, *Brevibacillus*, *Gordonia terrae*, *Chryseobacterium lathyri*, *Ensifer sesbaniae*, and *Paenibacillus illinoisensis* with high ability to dissolve P (65–496 mg/ L) and salt tolerance upto 1.5 M NaCl.

The improvement of soil structure and nutrient status of stressed soil by ST-PGPR is a major breakthrough and the most dependable bioremediation solution which can lead to agricultural sustainability.

2.3.2 Role of ST-PGPR in improving plant growth under salinity stress

An important concept of saline soil reclamation includes promotion of plant growth to rejuvenate the degraded quality of land. The highly depressed growth of plants in saline soils increases the risk of erosion because the salts are exposed to air, water and the absence of soil binding by roots enhances the evaporation, thereby hardening the land and marginalizing the fertility. Among the dynamic associations in the rhizosphere, healthy plant-microbe interaction is the most crucial factor deciphering the productivity of lands and in case of salty lands a bio-indicator of tolerance conversation between the microflora and plants. The accumulation of Na ions in plant tissues negatively affects growth and metabolism including reduced photosynthesis, membrane damage, stomatal closure, cell flaccidity, osmotic imbalance, ethylene stress and generation of reactive

oxygen species (ROS) leading to DNA mutation, membrane instability, protein degradation (Numan et al. 2018). The aforementioned role of microbes to ameliorate soil structure and fertility is a way of enhancing plant growth. Apart from this, the arrays of metabolites produced by PGPB confer tolerance in plants and increase their chances of survival.

2.3.2a Phytohormone production

The sessile nature of plants physiologically challenges them in responding to salinity stress. The decrementing phytohormonal levels under the inhospitable condition majorly ruin the growth of plants. Thus, addition of extrinsic factors including PGPR serves as the most feasible and environmental friendly solution to reprogram the plant metabolism and evoke the hormonal signalling (Khare et al. 2018). Indole acetic acid (IAA), a derivative of auxin diminishes the salt toxicity *via* activation of radicular systems, extending the apical meristem, improving the root and shoot structure thereby inciting root and stem associated physiological responses to combat salinity stress (Sachs 2005; Yu et al. 2020). Researchers have also notified increased endophyte colonization efficiency in the presence of auxins (Fouda et al. 2019). The combination of plant IAA and PGPR produced IAA initiates plant cell proliferation and elongation (Salwan et al. 2019). Hassan and Bano (2019) reported that inoculation of wheat with wild IAA producing *P. moraviensis* increased the rhizospheric and leaf IAA content by 33% and 30% respectively thereby enhancing the growth parameters whereas IAA-deficient mutants of *P. moraviensis* halted the growth in saline soil. The efficiency of *Curtobacterium albidum* strain SRV4 reported by Vimal et al. (2019) highlights that IAA production and other PGP traits by the strain improved the growth of rice, alleviating salinity stress. Next generation sequencing of *Sinorhizobium meliloti* also elucidated that in IAA overproducing strains the stress response genes were induced

modifying the tolerance and survival of bacteria under stressed conditions (Defez et al. 2016). IAA producing strain *Bacillus nanhaiensis* TY0307 led to 30% increase in rice shoot length under 9 g/L NaCl condition (Zhang et al. 2018). Relatively, Wang et al. (2021) found that combination of *Brevibacterium frigoritolerans, Bacillus velezensis* and *Bacillus thuringiensis* capable of producing IAA, siderophore and ACCD along with nitrogen fixation and phosphate solubilization potential, when applied to wheat plants under salt stress promoted the growth parameters of the plants by almost 2-folds (at 400 mM NaCl). Saleem et al. (2021) reported the production of indolic compounds including indole, indole-3-butyramide, benzylmalonic acid and 4-methyl-2-pyrrolidinone, by salt-tolerant strains of *Bacillus* sp. which was found to be increasing with salinity upto 500mM NaCl. The study also concluded that application of strains to *Gossypium hirsutum* under salt stress elevated the plants growth parameters, chlorophyll content, K^+ content in leaves, RWC and reduced Na^+ from soil. Gibberellic acid (GA) is another plant growth regulator responsible for cell division, hypocotyl elongation, increasing meristem size, ameliorating metabolic processes such as chlorophyll synthesis, antioxidant activity, preventing lipid peroxidation and photosynthetic rate (Maggio et al. 2010; Etesami and Beattie 2018; Etesami and Glick 2020). Kang et al. (2014) reported that gibberellin producing *P. putida* H2-3 regulated hormonal and stress physiology of soybean under salinity and drought stress. Isolating four salt-tolerant strains namely *Bacillus* sp., *A. brasilense, A. lipoferum,* and *P. stutzeri* with potential of producing four different phytohormones IAA, GA, cytokinins (CKs) and abscisic acid (ABA), upon application to wheat plants as consortium under salt stress of 150 mM NaCl, showed reduced electrolyte leakage and increased chlorophyll content, osmolyte accumulation and antioxidant activity (Ilyas et al. 2020).

ABA is a stress related hormone critically important in providing salt tolerance to plants by mediating expression of stress responsive genes (Ren et al. 2018). ABA upgrades the water content by ascending transpiration flow regulating stomatal aperture in leaves, protects against oxidative damage caused due to salt toxicity (Cutler et al. 2010). ABA also stimulates synthesis of strigolactones (SLs) which is a newly studied plant hormone playing significant role in architectural development of plant roots according to environmental hospitable and inhospitable conditions (Mishra et al. 2017). Hamayun et al. (2017) modulated the triggering of stress responsive genes in the presence of ABA synthesized by strain *P. spadiceum* AGH786 in salt affected soybean. Bharti et al. (2016) showed initiation of ABA-signalling cascades increasing expression of salinity mitigation genes in wheat when inoculated with halotolerant strain *Dietzia natronolimnaea* STR1. Saakre et al. (2017) revealed that application of *P. fluorescence* to rice plants elevated the intrinsic salt-tolerance level of plants due to triggering of ABA synthesis genes particularly during late reproductive stage. Woo et al. (2020) confirmed the action of PGPR strain *B. subtilis* strain GOT9 in conferring both drought and salinity stress resilience in *Arabidopsis thaliana* and *Brassica campestris* upon inoculation. The study illustrated that application of GOT9 to stressed plants elevated the expression of several ABA-inducible stress mitigating genes such as *RD29B, SOS1, RD29A, RAB18, RD20, WRKY8,* and *NCED3. RD29B and RAB18, RD29A* genes are members of dehydrin family and functionally linked to drought and salt tolerance (Yu et al. 2018; Arora et al. 2020b); while *RD29B*, *SOS1, RD29A,* and *WRKY8* are typical salt-stress inducible genes. The application henceforth caused lateral root growth and development and effectively minimized the damages in plants caused by both the stresses. The work also summarized that endogenous ABA level is highly significant in expression of salt-drought responsive genes and tolerance to both stresses.

2.3.2b Nutrient chelation

Apart from the phytohormonal modification, the action mechanism of ST-PGPR also involves chelation of nutrients from a nutritionally challenging environment. The translocation of nutrients by plants is crucially limited under salinity stress due to osmotic imbalance and lowered turgor pressure. In addition to improvement in soil nutrient status, PGPR also stimulate the uptake of essential minerals in plants. Correspondingly, Verma and Arora (2018) reported the mitigation of noxious salinity effects in *Crotalaria juncea* (Sunhemp) by inoculation with salt tolerant rhizobia *via* improvement in nitrogen fixation activity. Upadhyay and Singh (2015) found that strain *Bacillus aquimaris* increased NPK content in wheat leaves under saline stress. Fan et al. (2017) concluded that the treatment with mixture of *B. amyloliquefaciens* IN937a and *B. pumilus* T4 elevated the N uptake by 39% in *Lycopersicon esculentum* Mil. in comparison to merely 10.3% N uptake without PGPR when grown in calcareous sodic soils. *Azotobacter* and *Azospirillum* are characterized as potential biofertilizer with nitrogen fixing ability mitigating salinity stress (Akhter et al. 2012; Nag et al. 2018). Further, explaining P chelation in plants by ST-PGPR, Kadmiri et al. (2018) studied the role of IAA producing and inorganic phosphate solubilizing strain *P. fluorescens* Ms-01 chelating phosphate up to 22.6 µg/ ml in 600 mM NaCl and promoting plant growth both under normal and saline conditions. Saghafi et al. (2018) reported growth promotion of *B. napus* L. under inoculation with *Rhizobium sp.* in the presence of salinity and the increment of phosphorous content in shoot by about 24% in comparison to control. Selvakumar et al. (2018) showed that inoculation with bacteria *Pseudomonas koreensis* improved P content in maize root and shoot which were subjected to various salinity levels (0, 25, 50 mM NaCl). The chelation of iron by siderophore production is a prominent strategy in promoting plant growth in saline

stressed conditions (Panwar et al. 2016). Rosier et al. (2018) discussed the dual role of siderophores including nutrient stimulation in plants and eliciting stress associated defense responses diluting the toxicity. Viscardi et al. (2016) reported salt tolerance properties of *A. chroococcum* strains showing siderophore production along with other PGP traits alleviating the growth in tomato plants stressed by salinity. Mahmood et al. (2016) comparatively found that siderophore producing *Bacillus drentensis* P16 strain was a significant plant growth promoter improving salinity stress in mungbean in comparison to non-siderophore producing *Enterobacter cloacae* P6. Bhise et al. (2017) found that siderophore production by *Chryseobacterium gleum* sp. SUK promoted growth of rice stressed by salinity.

Availing zinc to the plants against a nutrient deficient environment is another mitigation strategy regulating the metabolic processes such as enzymatic activity, photosynthesis, gene expression, disease resistance, protein synthesis (Arora et al. 2020b). Zn solubilization activity by strain *Klebsiella sp*. IG 3 promoted growth of oats seedling under salt stress (Sapre et al. 2018). Eida et al. (2019) also isolated zinc solubilizing salt tolerant strains and showed their effect on growth promotion of various plants. Thus, the efficacy of salt tolerant microbes in availing the nutrients to stressed plants is a sustainable solution and lot more can be done on optimizing the conditions such as temperature, pH or use of selective additives to enhance the chelation rate of microbes.

2.3.2c Osmolyte accumulation

The primary line of defence by salt tolerant microbes includes the maintenance of osmotic balance by accumulating low molecular weight soluble sugars, amino acids, quarternary amines, tetrahydropyrimidines and imino acids called as osmolytes/ osmoprotectants/ compatible solutes (Mishra et al. 2018; Fatima and Arora 2019). Although plants also produce osmoprotectants but consume more energy and the

synthesis penalties plant biomass. Thus, to compensate the excessive energy consumption by plants, the extrinsically applied PGPB divide the energy loss and combat the salt toxicity without degrading the plant health. Osmoprotectants regulate ionic equilibrium across the membrane, protects denaturation of proteins and macromolecules (like DNA, RNA), chaperons the folding of proteins, and stabilizes the turgor pressure (Tewari and Arora 2013; Paul and Lade 2014; Gong et al. 2020). The uptake of osmoprotectants synthesized by microbes follow proper channels where the accumulated osmolytes are released in the environment and finally are actively up taken *via* stretched activated channels, aquaporins and efflux systems (Miller and Wood 1996). Singh et al. (2017) concluded that salt tolerant PGP strain *E. cloacae* SBP-8 possessed osmolyte accumulation activity with up-regulation of osmoprotectant genes, promoting growth of wheat under high salinity (200 mM). The elevation of proline biosynthesis and upregulation of stress responsive genes CAPIP2, CaKR1, CaOSM1, and CAChi2 in capsicum was reported upon inoculation with *Bacillus fortis* SSB21 under salt stress (Yasin et al. 2018). Similarly, Hmaeid et al. (2019) found elevation in chlorophyll content, osmolytes and antioxidant activities in *Sulla carnosa* upon addition of *Acinetobacter sp.* (Br3), *P. putida* (Br18) and *Curtobacterium* sp. (Br20) under salt stress. Ilyas et al. (2020) found that with increase in salinity the proline accumulation by *Bacillus* sp. (KF719179), *Azospirillum brasilense* (KJ194586), *Azospirillum lipoferum* (KJ434039), and *Pseudomonas stutzeri* (KJ685889) elevated with highest level at 10% NaCl.

The application of bacteria consortia was also found to be effective in alleviating salt stress in wheat. Fatima and Arora (2021) also showed enhanced level of chlorophyll, flavonoid, phenolic, proline, protein and carbohydrate content along with increment in salt-tolerance properties of sunflower (such as antioxidant activity, reduction potential)

upon inoculation with EPS and *Pseudomonas entomophila* PE3. Pan et al. (2019) through meta-analysis studied the effect of PGPR inoculation on salt-sensitive and salt-resistant plants and found that the inoculation increased the osmolyte content of both the plants in salty conditions. Singh and Jha (2016) also reported modulation of osmoprotectant concentration (mainly soluble sugars) in chickpea under inoculation with *Serratia marcescens* CDP-13 in the presence of salinity. On the contrary grounds, some studies emphasize that under microbial inoculation, osmoprotectants (mainly proline) decrease in plants. Thus, the absence of equivocal view widens the area of research to exploit the exact action mechanism of osmoprotectants or enquire whether the accumulation of different osmolytes is genus/ species specific, or stress specific or even host specific. Interestingly, Xiong et al. (2020) established a relationship between halophytes and recruitment of salt-tolerant rhizospheric microbes to trigger resilience in plants under saline conditions. The study elaborated that root exudation by halophyte *Limonium sinense* (including 2-methylbutyric acid, stearic acid, palmitic acid, palmitoleic acid, and oleic acid) facilitated employment of PGP strain *Bacillus flexus* KLBMP 4941 to the plants. In return the salt tolerant strain regulated osmolyte contents, enhanced flavonoid and antioxidant enzymes, and maintained Na^+/K^+ homeostasis along with improvement in photosynthetic activity and growth of plants. Similarly, there was increment in soluble protein content, proline concentration, CAT, APX and GR activities in soybean plant under salt stress when the plants were treated with *B. subtilis* and *P. fluorescens* under salt stress (Abulfaraj and Jalal 2021).

2.3.2d EPS production

The survival of salt tolerant microbes and halophytes under hypertonic environment requires the synthesis of most important secondary metabolite i.e. EPS. The ecological, biochemical and physiological functionalities of EPS distinguish them as stress

insulators against an osmotically challenged surrounding. The retrogression of microbes from planktonic to sessile stage, on being exposed to salinity, mandates the necessity of an organo-mineral sheath (biofilm) to confine the microbial cells in nutrient and water saturated micro-environment (Qurashi and Sabri 2012). EPS also mantles the rhizoplane protecting the association of plant roots and microbes, which serves as an active site for nutrient cycling, nutrient chelation, water uptake, osmotic adjustments, nodulation and cationic exchange (Bhargava et al. 2016). The micro-ecosystem in EPS under stress conditions holds triadic amalgamation including cohesive bonding among the microbes and adhesive bonding between the microbes, plants and the soil nutrients. This stable and osmotic-shock proof consortium defines the most successful stress response by microbes to safeguard the survival of both plants and microbes against salinity and water stresses.

Gu et al. (2017) optimized and characterized the glycan type EPS produced by halobacterium *Kocuria rosea* ZJUQH from Chaka Salt Lake and declared the treatment of saline soils using this metabolite. Interestingly, Boukhelata et al. (2019) correlated the rheological properties of EPS with water absorption potential. The study concluded that the rheological properties of EPS-R1 produced by Saharan bacterium *Paenibacillus tarimensis* REG 0201M were dependent on molecular weight of the polymer. The viscosity parameters of extracted EPS supported 3 to 5 times more water adsorption than xanthan (standard polymer used in industries). Relatively, Niu et al. (2018) found that isolate *P. fluorescens* DR7 with highest EPS production ability responded best under drought conditions upon inoculation to foxtail millet, in comparison to isolate DR11 with highest ACC deaminase activity. Further only isolate DR7 was able to increase the RAS/ root tissue (RT) ratio and soil moisture while DR11 had no impact on water availability and soil reclamation. Therefore, EPS production was concluded

as the key player in promoting the growth of plants under water stressed conditions comparing with other PGP traits. Fathalla (2018) reported significant biofilming and EPS producing activity at 0, 50, 100 and 150 mM NaCl by strain *Pseudomonas anguilliseptica* SAW 24 which thereby showed highest records of plant growth promotion in *Vicia faba*. Tewari and Arora (2014a, b, 2016) studied the efficacy of talc based EPS bioformulation of *P. aeruginosa* in enhancing the growth and salt resistance in sunflower grown in saline regions. Naseem et al. (2018) reported that EPS producing PGPR upon inoculation promote the accumulation of proline, free amino acids and sugars in plants under water deficit conditions. Hossain et al. (2016) correlated the salinity tolerance index in mung bean with EPS produced by applied bacteria and found a positive relation between the two. There is strong correlation between salinity and EPS production by microbes and Zeng et al. (2016) reported salinity as the limiting factor for EPS production. The study found that EPS production by *Rhodopseudomonas acidophila* increased with change in NaCl concentration from 0 to 10.0 g/L, and the metabolite was the main reason for increase in microbial biomass and cell survival.

Biofilming by *Brevibacterium halotolerans* FAB3 in wheat under various saline conditions (125, 250, 500 mM NaCl) improved the growth parameters and yield of the inoculated plants. The biofilming activity of the isolates were enhanced by 31.7 and 32.6% at 125 mM and 250 mM NaCl conditions respectively and even the EPS production increased at 125 mM in comparison to 0 and 75 mM NaCl conditions (Ansari and Ahmad 2018). Fatima et al. (2020) determining the primary salinity response of *Alcaligenes* sp. AF7 found that EPS production was the most dominating property with exponential increase in production from 0 to 300 mM NaCl. Interestingly the study also found direct correlation between EPS production and Na^+ chelation and proline accumulation and resultantly the direct application of EPS along with AF7

stimulated the growth of rice grown in saline condition. Nunkaew et al. (2015) supported the essentiality of EPS in microbial survival as the macromolecule is the key factor to bind Na^+ reducing the toxicity in plants and bacteria. Further extending the complex relationship between salinity and EPS production,

Fang et al. (2018) elucidated that salinity is the major factor for inducing changes in the composition of EPS. Along with the direct involvement in osmoprotection and salt chelation, the components of EPS also play significant role in stress mitigation. For example, the carbon reservoir of EPS serve as energy source, protein content helps in maintaining membrane stability and phenolics mediate seed germination and metabolic activities (Kavi Kishor et al. 2005; Sandhya et al. 2009; Sharma et al. 2019). The functionality of EPS under saline conditions also includes the ability to reduce ROS through their antioxidant activity, reduction and hydrogen peroxide scavenging potential (Kohler et al. 2009). Abdhul et al. (2014) delineated that with the increasing concentration of free radicals the scavenging ability of EPS also increased linearly. Liu et al. (2017) explained that EPS was the critical parameter in the survival of *M. alhagi* CCNWXJ12 – 2^T under salt stress, where the salt-resistant property of the wild strain was higher than the EPS-defective mutant strain which (the latter) showed elevated cellular Na^+ content, thereby desiccating the cell. Kasotia et al. (2016) concluded that the amount of EPS produced by *Pseudomonas* strain AK1 increased from 0 mM to 300 mM NaCl and with the increase in EPS production, the EC_e of the inoculated plants decreased to 0.9 dS/m due to the binding of EPS to Na^+. Yaakop et al. (2016) also observed elevated expression of EPS gene clusters at 20% (w/v) NaCl. Ashraf et al. (2004) reported the apoplastic flow of sodium ions across the plant roots to leaves, which was restricted under the inoculation of EPS producing bacteria through their biofilming action, effluxing the Na^+ ions. Similarly, Mukherjee et al. (2019) reported

that EPS extracted from *Halomonas* sp. Exo1 induced chelation of Na^+ through the presence of uronic acids. The study also showed that the antioxidant property and sodium chelation helped in promoting growth of rice upon direct inoculation under salt-stress.

Therefore, it can be concluded that the fate of salt tolerant bacteria is highly dependent on the production of EPS. The complete exploration of this secondary metabolite is still pending and also the responsive links it has with other metabolites or their associated genes.

2.3.2e Scavenging of ROS

Abiotic stress such as salinity disrupts cellular metabolic homeostasis and leads to production of ROS such superoxide radical (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2). This oxidative stress damages the membrane protein and lipids, nucleic acids, metabolic enzymes, activates programmed cell death (PCD), inhibits photosynthesis and photo-chemical processes (Abogadallah 2010; Chiappero et al. 2021). The negative impact of ROS on nodules represses the tissue integrity, nitrogenase activity and the functionality of leghemoglobin. This impaired leghemoglobin activity leads to poor bacteroid formation and cell respiration (Mhadhbi et al. 2015). Although plants elicit a series of antioxidant enzyme synthesis, yet inoculation with PGPR supports in reducing the energy demand of plants involved in activating the antioxidant systems. The ROS scavenging activity by PGPR includes synthesis of various antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), polyphenol oxidase, guaiacol peroxidase, monodehydroascorbate dehydrogenase (MDHAR), dehydroascorbate reductase (DHAR) (Chawla et al. 2013). Apart from these, PGPB

also synthesize ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics as the non-enzymatic ROS scavenging antioxidants (Sharma et al. 2012).

Sukweenadhi et al. (2018) showed that the priming of *Panax ginseng* seeds with *Paenibacillus yonginensis* DCY84^{T} induced the expression of several antioxidant genes, including *PgAPX* and *PgCAT* recovering the plant from salt stress. Santos et al. (2018) also elaborated that the highest accumulation of ascorbate (non-enzymatic antioxidants) was in cowpea nodules inoculated with *Bradyrhizobium under salt stress.* The study also elucidated that the co-inoculation of cowpea with *Bradyrhizobium* and *Trichoderma* showed the highest accumulation of total glutathione (5.1 µmol g^{-1} DW) in saline conditions. He et al. (2021) found that inoculation of ryegrass (*Lolium perenne* L.) with *Bacillus* sp. WM13-24 and *Pseudomonas* sp. M30-35 under water stress conditions, after 7 days increased the photosynthetic capacity, RWC, catalase (CAT) and peroxidase (POD) activities and proline content of plants and additionally decreased the malondialdehyde (MDA) content, relative membrane permeability (RMP) and H_2O_2 accumulation along with reduction in ABA content in leaves. Similarly, another study by Desoky et al. (2020) observed significant improvement in physiological attributes of *T. aestivum* plants along with elevated antioxidant activities, while levels of Na^+ and oxidative stress biomarkers (*e.g.*, $O_2^{\bullet-}$ and H_2O_2) were highly reduced, upon inoculation with *B. cereus, S. marcescens,* and *P. aeruginosa* under saline conditions (150 mM and 300 mM NaCl).

The reduction of ROS by the antioxidant systems is an intern capability of plants but the inoculation with PGPR provides a resonating improvement in stress responses and growth of plants.

2.3.2f ACC deaminase production

Under salt stress, the shooting ethylene levels in plants through triple chronological events negatively influence the seedling length, shoot diameter and directional change of growth (Glick et al. 2007). Although ethylene is a physiologically important hormone for plants, but the peaking ethylene concentration in response to any climate change or stress highly depresses the growth of plants and if not monitored leads to plant death. ACC is the immediate precursor of ethylene and the action substrate for PGPR. Beneficial microbes lower the ethylene level *via* production of ACCD which hydrolyses ACC to α-ketoglutaraldehyde along with supplementation of energy (Gamalero and Glick 2015). The action of ACCD is in correspondence to the two peaks of ethylene accumulation in plants. The first small peak of ethylene induced by ACC oxidase elicits the transcription of protective or defensive genes. The second large peak of ethylene accumulation induced by ACC synthase, triggers the action of bacteria producing ACCD through lowering of ethylene by about 50-90%. The only resistance in the action of bacterial ACCD is the comparatively stronger affinity of ACC oxidase for ACC. Thus, ACCD must bind before significant ACC oxidase is produced (Glick 2014).

Rhizobia with ACC deaminase activity are found to be 40% more efficient in forming nitrogen fixing nodules than those without the enzymatic activity (Ma et al. 2003, 2004). Timmusk et al. (2011) assessed the selective pressure for bacteria with ACC deaminase and found that 50% of bacterial population isolated from South Facing Slope in Northern Israel, where the climatic conditions were adverse with excessive sunlight and frequent drought had ACC deaminase activity; whereas only 4% had the activity in northern Israel where the climatic conditions were hospitable. Various novel halotolerant diazotrophic strains including *Brachybacterium saurashtrense* sp. nov.,

Zhihengliuella sp., *Brevibacterium casei*, *Haererehalobacter* sp., *Halomonas* sp., *Vibrio* sp., *Cronobacter sakazakii*, *Pseudomonas* spp., *Rhizobium radiobacter*, and *Mesorhizobium* sp. isolated from the rhizosphere of extreme halophyte *Salicornia brachiate* showed potent phytohormonal production, nitrogen fixation, nutrient chelation and ACCD activity (Jha et al. 2012). Fan et al. (2020) isolated four endophytic strains belonging to genera *Pseudomonas*, *Bacillus*, *Mucilaginibacter* and *Rhizobium* from *Phalaris arundinacea*, *Solanum dulcamara*, *Scorzoneroides autumnalis*, and *Glycine max*, respectively and applied to *A. thaliana* plants under salt stress (200 mM NaCl). The primed plants showed elevated growth parameters, antioxidant capacity, leaf chlorophyll concentration and photosynthetic activity. The study concluded that the exhibition of ACCD activity by bacteria helped to balance the ethylene level in plants thereby protecting the photosynthetic apparatus from ROS. Additionally, growth regulators (IAA, GA, CKs) synthesized by the strains promoted growth; GFP tagging also revealed the strains were successfully colonizing the roots of the plants.

Employing various mechanisms and actions against the odds of salinity, ST-PGPR mitigate the gradual waning in crop productivity. With emerging collection of ST-PGPR in various research labs globally, bioinoculants have been developed and are available in market for public use. For example, Rizos® developed using strain *B. subtilis* UFPEDA 764, stimulates metabolite production, germination rate, plant growth parameters and improves the stress resistance and yield of plants (Lallemand; http://labfarroupilha.com/); Onix®, associated strain *B. methylotrophicus* UFPEDA 20, utilized to promote growth of plants under abiotic stress such as salinity (Lallemand; http://labfarroupilha.com/); Azos®, associated microbe *A. brasilense* abv-5, the product promotes plant growth under water stress by decreasing the concentration of ethylene,

thereby elevating photosynthetic activity, also the bioinoculant enhances the absorption rate of water and nutrients in plants (Arora et al. 2020a; Lallemand; http://labfarroupilha.com/); TNC Bactorr^{S13} (The Nutrient Company; containing *Bacillus spp.*), Flortis Micorrize (Flortis®; containing mycorrhiza, *Tricoderma* along with *B. subtilis* and *Streptomyces* spp.) promote growth and resilience in plants under salinity (Miceli et al. 2021). The development of microbial products are showing promising results, however the technology needs further advancement and large scale application strategy to solve the problem of salinity in agroecosystem. Future studies to fine-tune the role of microbial metabolites in amelioration of salinity and their addition in bioformulations could be the new dawn of sustainable agriculture and microbial technology.

MATERIALS
&
METHODS

3.1 Isolation of salt-tolerant bacteria

Bacteria were isolated from semi-arid regions of Kanpur Dehat, (20°38 E and 80°21 N) in central Uttar Pradesh, India. White salt-crust of soil samples from a depth of 10-12 inches were collected in sterilized polythene bags. The chemical properties of the soil (from various sampling sites) including pH, electrical conductivity, organic carbon, organic matter, microbial biomass, available nitrogen, phosphorous and potassium were checked according to Jackson (1973) and Zhang et al. (2019b). Diluting plating technique, using nutrient agar (NA; Hi-media) containing 2% NaCl (w/v) was used to isolate salt-tolerant bacteria from the soil (soil samples serially diluted 10^{-5} to 10^{-6}) and incubating the plate at 28°C for 24 hours (Camargo et al. 2003). Various isolates were maintained on NA and checked for fluorescence on Pseudomonas Agar (for fluorescein; Hi-media) containing g per liter distilled water (DW): tryptone, 10; proteose peptone, 10; dipotassium hydrogen phosphate, 1.5; magnesium sulphate, 1.5; agar, 15; final pH (at 25°C) 7.0 ± 0.2 (MacFaddin 1985). Fluorescence of the colonies was also detected under UV light using Transilluminator (GENEI UV- Transilluminator Model MD-20). Isolates were selected on the basis of their salt-tolerance level and pure cultures were preserved in 25 % glycerol stock at − 20ºC (Labocon, LUF-86-100) for further use.

3.2 Phenotypic characterization

Colony morphology of the isolates were examined for growth pattern, color, shape, size, elevations and texture by observing growth on NA and Pseudomonas Agar (for fluorescein) (Khandelwal 2002). Cell morphology was detected through Gram's staining (Gram 1884). Motility of the isolates were determined by observing the growth pattern on semi-solid motility test-agar media (Tittsler and Sandholzer 1936).

3.3 Biochemical and physiological properties

The isolates were further examined for the biochemical and physiological characters as per Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and Bergey's Manual of Systematic Bacteriology (Garrity 2005). Standard protocols were followed for each tests.

(i) Generation time:

Growth kinetics of the isolates was estimated following the protocol of Dubey and Maheshwari (2012). The log phase culture of the isolates (OD 610 = 0.1) was inoculated in 50 ml of nutrient broth (NB) and incubated at 25 ± 2°C for 48 hours at 120 rpm. Samples were withdrawn after every 4 hours and the optical density was measured at 610 nm using Thermo Scientifc™ Evolution 201 UV–Vis spectrophotometer. The generation time was calculated using the formula:

Generation time = (T2–T1)/ 3.3 (log10 OD2- log10 OD1)

where (T2-T1) = difference of two time intervals at any two point in log phase in growth curve; (log10 OD2- log10 OD1) = difference between the log10 value of OD2 at time T2 and OD1 at time T1

(ii) Growth at different temperature:

The inoculated NA plates (using log phase culture; OD 610 adjusted to 1) were subjected to various temperature conditions ranging from 4 to 45°C and after incubation for 120 hours the growth was observed (Pandey et al. 2006).

(iii) Growth at different pH:

The pH tolerance of the selected isolates was done according to Zablotowicz and Focht (1981), by varying the pH range from 5.0 to 12. NA was used to determine the tolerance ability and pH was adjusted by addition of HCl or NaOH and the inoculated (using log

phase culture; OD 610 adjusted to 1) medium was incubated for 25 ± 2°C for 3-5 days and observed for growth.

(iv) Salt tolerance assay:

The halo-tolerance assay of the selected isolates was conducted using NB supplemented with 2–8 % NaCl (w/v) and non-saline control. The log phase culture (OD 610 adjusted to 0.1) of the isolates were added to medium and incubated at 25 ± 2°C with shaking at 120 rpm for 48 h. The optical density (at 610 nm) was measured using Thermo Scientific™ Evolution 201 UV–vis spectrophotometer after every four hours up to stationary phase and salt-tolerance curve was drawn (Khare et al. 2011). The experiment was conducted in triplicates and under same physiological conditions.

(v) Utilization of different carbon sources:

The isolates were checked for utilization of various carbon sources such as mannitol, glucose, fructose, lactose, dextrose, sucrose, maltose, malate, starch, glycerol, malate, trehalose and citrate. In the test, minimal medium M9 (5X) was used to grow the isolates and different carbon sources were added to check for their utilization (Jovčić et al. 2010). Different carbon sources (sterilized by filtration through Millipore membranes pore size 0.22 μm) were added to medium at concentration of 10% (w/v). The plates were incubated for 28 to 48 hours at 25 ± 2°C and were checked for growth.

(vi) Utilization of different nitrogen sources:

Different nitrogen sources such as yeast extract, potassium nitrate (KNO3), sodium nitrate ($NaNO_3$), tryptophan, ammonium chloride (NH_4Cl), glycine, ammonium sulphate ($(NH_4)_2SO4$), lysine, glutamine, cysteine, and methionine were added to minimal medium M9 (5X) in place of ammonium chloride (source of nitrogen in the

medium) (Holt et al. 1994). Streaked plates were incubated for 24 to 48 hours at 25 ± 2°C and growth was observed.

(vii) Catalase test:

Catalase test was performed by placing a loopful of culture on glass-slides and 2-3 drops of 3% hydrogen peroxide were poured over the colony and observed for effervescence due to release of oxygen (Graham and Parker 1964).

(viii) Oxidase test:

Oxidase activity was checked using oxidase disc saturated with Kovac's reagent (1% aqueous tetra methyl-para-phenylene-diamine solution) (Kovacs 1956). Fresh culture of the isolate was spotted on filter paper soaked with reagent. The appearance of dark purple color within 10 seconds of inoculation showed positive result.

(ix) Lactose test:

Prepared lactose agar medium plate was spot inoculated with isolates and after incubation for 24-48 hours at 25 ± 2°C, the plate was flooded with Benedict's reagent. The appearance of yellow colored zone around the colonies showed positive result for lactose fermentation (Naseem and Bano 2014).

(x) Growth on glucose peptone agar (GPA):

GPA medium plate was streaked with bacterial isolates and incubated at 25 ± 2°C for 48 hours. Bacterial growth showed positive result for the test (Singh et al. 2008).

(xi) Nitrate reductase activity:

Bacterial isolates were inoculated in tryptone yeast extract (TYE) medium containing 0.1 % KNO_3 (Campbell 1999). After incubating the medium for 48 hours at 25 ± 2°C

sulphanilic acid (8 g/l) in 5M acetic acid and α-naphthylamine (5g/l in 5M acetic acid) were added to each tubes. Color change was observed.

(xii) Urease test:

NA plates with added 2% (w/v) urea and 0.012% (w/v) phenol red were used for the test. Bacterial isolates were spot inoculated on the plates and incubated for 48 hours at 25 ± 2°C. Color change of the media from yellow to pink-red was observed for positive result (MacFaddin 2000).

(xiii) Gelatinase test:

Gelatinase medium containing gelatin was equally poured in test tubes. The tubes were stab inoculated with inoculating needle and the tubes were incubated for 3-5 days at 25 ± 2°C. After incubation the tubes were placed in refrigerator at 4°C for 15 minutes. Liquid state of the medium confirmed the positive result (McDade and Weaver 1959).

(xiv) Citrate utilization test:

Simmon's citrate agar medium plates were prepared and bacterial isolates were streaked and incubated for 24 hours at 25 ± 2°C. Color change from blue to green confirmed the positive result for the test (MacWilliams 2009).

(xv) Amylase test:

Starch agar medium was prepared and isolates were streaked on the plates and incubated for 48 hours at 25 ± 2°C. After incubation the plates were flooded with Gram's iodine and development of clear zone around the colonies was observed (Lennette et al. 1985).

(xvi) Lipase test:

Tween 80 agar medium was prepared, inoculated and incubated at 25 ± 2°C for 2-3 days. Opaque zone formation around the colonies was regarded positive (Samad et al. 1989).

(xvii) Protease test:

For this test skim milk agar medium was used and prepared plates were inoculated with bacteria. The plates were incubated at 25 ± 2°C for 48 hours. Clear zone around the colonies was observed (Kasana et al. 2011).

(xviii) Cellulase test:

Czapek's mineral salt agar medium plates were inoculated and incubated for 2-3 days at 25 ± 2°C. After incubation the plate was stained with congo red dye for 15 minutes and 1% NaCl for destaining for 15 minutes. Halo zone around the colonies was observed (Florencio et al. 2012).

(xix) Ammonia production:

Peptone broth was prepared and 5 ml of the medium was added to various tubes. Bacterial culture was inoculated to each tube and incubated for 48 hours at 25 ± 2°C. After incubation 0.5 ml of Nessler's reagent was added to each tube and observed for color change along with slight precipitation (Cappuccino and Sherman 1992).

(xx) Indole test:

Prepared tryptone broth was inoculated with bacterial culture and incubated for 24 hours at 25 ± 2°C. After incubation, 1 ml of Kovac's reagent was added to each tube and formation of "red color ring" on top of the broth was observed (Cheesbrough 1985).

(xxi) Methyl Red-Voges Proskauer (MR-VP) test:

MRVP broth was prepared and 10 ml was dispensed in various tubes. Each tube was inoculated with bacterial culture and incubated for 28 hours at 25 ± 2°C. After incubation, media was divided into two tubes one for MR and the other for VP test. For MR test 5 drops of methyl red and for VP test 12-15 drops of VP- I (5% α- naphthol) and VP- II (40% potassium hydroxide) were added to each set of tubes. Color change was observed after keeping the tubes for 10-15 minutes, (Faddin and Jean 2000).

(xxii) Polyhydroxy butyrate (PHB) production:

Bacterial culture was streaked on NA plates and incubated at 25 ± 2°C for 48 hours. After incubation ethanolic solution of 0.3% (w/v in 70% ethanol) Sudan Black B dye was spread over the colonies for 30 minutes for staining and was destained by washing with 96% ethanol to remove extra stain. The colonies still retaining Sudan Black B dye were considered to be positive for PHB accumulation (Kitamura and Doi 1994).

(xxiii) H_2S (hydrogen sulphide) production:

Sulphite indole motility (SIM) medium was used to detect H_2S production by bacteria. The containing SIM agar medium were stab inoculated with isolates and incubated for 24-48 hours at 25 ± 2°C. Color change of the medium to black was observed and regarded as positive for H_2S production (Cappuccino and Sherman 2008).

3.4 Plant growth promoting properties under various saline conditions

Further, on the basis of PGP properties, two strains showing best results were selected and characterized in the presence of salts (2% to 8% NaCl).

Prior to the quantification of PGP properties of isolates, homogenous bacterial suspension were prepared by inoculating log phase culture (24 hrs old with OD 610 adjusted to 0.1, CFU 10^8 cells/ ml) to specific media (according to the protocols). The

culture filtrate was obtained by centrifugation at 10,000 rpm (*13,081 x g*) (21ºC) and further filtered through 0.45 µm membrane-filter (Merck, India) (Khare et al. 2011).

3.4.1 ***Phosphate solubilization***

Quantitative phosphate solubilization (tricalcium phosphate) was conducted using colorimetric assay according to Watanabe and Olsen (1965). PKV broth (50 ml) supplemented with various salt-concentrations was prepared in Erlenmeyer's flask. Homogenous bacterial suspension was added to each flask and then incubated for 5 days at 25 ± 2°C and 120 rpm. Uninoculated medium served as control. After incubation, the cultures were centrifuged at 11,000 rpm *(14050 x g)* for 10 minutes. Now, 1 ml of culture supernatant was mixed with 9 ml of autoclaved distilled water in tubes and 2.5 ml of color reagent was added to each tube. The reagent was freshly prepared by mixing solution A and B and maintaining to 2L; Solution A: 12g ammonium molybdate $\{(NH_4)_6Mo_7.4H_2O\}$ + 250 mL distilled water; Solution B: 0.2908 mg antimony potassium tartrate $\{K(SbO)C_4H_4O_6.1/2\ H_2O\}$ in 1000 mL of 5N H_2SO_4(148 mL conc.$H_2SO_4\ L^{-1}$)]. To 140 ml of the reagent 0.74 g ascorbic acid was added slowly and gently stirred. The development of blue color was observed and the optical density was measured at 880 nm. The amount of phosphorous solubilized was determined using known molarities of potassium di-hydrogen phosphate (1-200 µM).

3.4.2 ***Zinc solubilization***

Zinc solubilization property of the isolates was checked on zinc media (dextrose, 1%; ammonium sulphate, 0.1 %; potassium chloride, 0.02 %; di-potassium phosphate, 0.01 %; magnesium sulphate, 0.02 %; zinc oxide, 0.1 %; agar, 3%) with supplemented NaCl (2-8%) and non-saline control. The plates were inoculated with bacteria and incubated

for 48 hours at 25 ± 2°C (Fasim et al. 2002). The zinc solubilization index (ZSI) was calculated by using the formula:

ZSI = (Colony diameter + halo zone diameter)/ Colony diameter

3.4.3 *Potassium solubilization*

The ability of bacteria to chelate potassium from insoluble source (mica/ potassium aluminium silicate; $KAl_2[AlSi_3O_{10}](OH)_2$) was checked on Aleksandrov medium plates (glucose, 5g; $MgSO_4.7H_2O$, 0.5 g; $CaCO_3$, 0.1 g; $FeCl_3$, 0.006 g; $Ca_3(PO_4)_2$, 2g; mica, 3g; agar, 20g; 1L distilled water) with supplemented NaCl (2-8% NaCl; non-salinized control). The media was modified by adding acid-base phenol red indicator dye (100 mg/ L). The inoculated plates were incubated for 3-5 days at 25 ± 2°C and the change in color and formation of zone around the colonies was observed (Rajawat et al. 2016).

3.4.4 *Siderophore production*

Detection of siderophore production by isolates were checked using the standard CAS (chrome-azurol sulfonate) assay (Schwyn and Neilands 1987). The glassware was priorly washed with 3M HCl to remove traces of iron and then rinsed with sterilized distilled water (Cabaj and Kosakowska 2007). CAS reagent was prepared using the protocol of Schwyn and Neilands (1987). CAS dye 121 mg, was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($FeCl_3.6H_2O$) solution was prepared in 10 mM HCl. Subsequently, the solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 ml distilled water) with continuous stirring and was sterilized before use. Both qualitative and quantitative estimation of siderophore production was estimated using following protocols:

Qualitative analysis was done on CAS plates, prepared by adding 100 ml of CAS reagent to 900 ml Luria Bertani (LB) agar medium (Hu and Xu (2011) supplemented with NaCl (2-8%) and non-salinized plate for comparison. The plates were spot

inoculated with isolates and were left for incubation for 5-7 days at 25 ± 2°C. The development of yellow to orange colored zone around the colonies indicated siderophore production.

Quantitative estimation of siderophore production was done as per CAS-Shuttle assay (Arora and Verma 2017). The bacteria were priorly grown in LB broth (with added NaCl at different concentrations) and the supernatant was collected by centrifugation at 10,000 rpm (*13,081 x g*) (21ºC). The supernatant of each isolate (0.5 ml) was mixed with equal amount of CAS reagent (0.5 ml) and after 20 min OD was taken at 630 nm (Thermo Scientific, Evolution 201).

The amount of siderophore produced was calculated as percent siderophore unit (psu) using the formula:

Siderophore production (psu) = {(Ar-As)/ Ar} x100

where Ar = absorbance of reference (CAS solution and un-inoculated broth), As = absorbance of sample (CAS solution and cell free supernatant of sample)

3.4.5 *IAA production*

Synthesis of IAA was checked according to Ahmad et al. (2008) by colorimetric assay using Salkowski reagent. The isolates were grown in NB amended with 0.5% tryptophan and various NaCl concentrations (2-8%) along with non-salinized control. After incubation the supernatant was collected by centrifugation at 10,000 rpm (*13,081 x g*) for 30 min. Salkowaski reagent was prepared by adding 1.0 ml of 0.5 M ferric chloride ($FeCl_3$) to 50 ml of 35% perchloric acid. Subsequently, 2-3 drops of orthophosphoric acid were added to 2 ml of culture supernatant, followed by addition of 4 ml Salkowaski reagent. The development of pink color was quantified by measuring the optical density at 530 nm and the value was calculated using the standard IAA (concentration 10–100 µg/ ml; Hi-media).

3.4.6 *Gibberellic acid production*

GA production was conducted using the spectrophotometric method described by Paleg (1965). Bacterial cultures were grown in NB medium supplemented with NaCl (2-8%) and non-salinized control. The culture supernatant was collected through centrifugation and 25 ml of the filtrate was dispensed in each tubes. To each tube 2 ml of zinc acetate (21.9 g zinc acetate with 1 ml glacial acetic acid and volume made upto 100 ml using sterilized distilled water) was added. After 2 minutes, 2 ml of potassium ferrocyanide was added and the mixture was again centrifuged at 2000 rpm (*523 x g*) for 15 min. The collected supernatant (5 ml) was added to equal amount of 30% HCl (5 ml) and incubated for 75 mins at 20^{o}C. Diluted HCl (30% v/v) was used as blank sample (5 ml) and uninoculated tube was taken as negative control. The optical density of the tubes were taken at 254 nm and standard curve was prepared using GA (Hi-media, 99 % pure) (concentration range 100− 1000 µg/mL).

3.4.7 *Hydrogen cyanide production*

HCN production by bacteria was checked according to the modified protocols of Bakker and Schippers (1987) and Sadasivam and Manickam (1992). The bacteria were grown on NA medium amended with glycine (4.4 g/ l) and supplemented with NaCl (2-8%) along with non-saline control. Uniform strips of filter paper (Whatman No. 1) (10 x 0.5 cm^2) dipped in alkaline picrate solution were hanged inside the test-tube containing inoculated medium. The tubes were incubated for 48 h at 25 ± 2^{o}C and reduction of sodium picrate forming reddish-brown color was concluded as positive for HCN production.

3.4.8 *Biocontrol activity*

Antagonistic activity of the bacteria was checked against potent fungal phytopathogens (*Macrophomina phaseolina, Fusarium solani, Fusarium oxysporum, Alternaria*

brassicae). Phytopathogens were obtained from Culture Collection of Rhizosphere Microbiology and Plant-Microbe Interaction Laboratory, School of Earth and Environmental Sciences, BBA University, Lucknow (Uttar Pradesh, India). The fungal culture were maintained on potato dextrose agar (PDA) (Hi-media, Mumbai) at 4°C. The antagonistic property of all the isolates were checked by dual culture technique following the standard protocol of Ikediugwu and Webster (1970). The disc (~6mm) from actively growing fungal plate was excised and placed on the center of PDA plates. Exponentially growing bacterial culture were single streaked on the either side of the PDA plate (equidistant from periphery) and incubated at 28°C for 5 days. The control plate was inoculated only with fungus. The radial growth of the fungus was measured in control and bacterial culture inoculated plate.

The percent inhibition was calculated using the formula:

$$\mathbf{L= (C-T/ C) \times 100}$$

where L= percent inhibition of radial mycelial growth, C= radial growth of the pathogen in the control, T= radial growth of the pathogen in the presence of isolated strains

3.4.9 *Exopolysaccharide production*

The extraction and purification of EPS was done by growing the isolate in NB (supplemented with 2-8% NaCl and non-salinized control) for 48 h at 200 rpm shaking and 25ºC. After incubation the cell free supernatant was obtained by centrifugation (7000 rpm (*6407 x g*) at 4ºC, for 20 minutes). The extraction of polymer was done using chilled acetone (1:3 ratio), following the standard method of Mody et al. (1989). Finally the extracted crude EPS was dialyzed using dialysis bag (12 to 14 kDa) against distilled water at 4ºC for 48 h in order to remove the extra proteins, unwanted monosaccharides and NaCl (in case of salt-exposed EPS). The purified EPS was dried

at 35ºC for 48 h and the weight of EPS was expressed as g/ 100 ml (Mendi and Aslim 2014).

3.4.10 *Qualitative estimation of ACC deaminase activity*

For checking the ACCD activity by isolates, sterile minimal DF (Dworkin and Foster) salts media (DF salts per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH 7.2) amended with 3 mM ACC instead of $(NH_4)_2SO_4$ as sole nitrogen source was used (Dworkin and Foster 1958; Penrose and Glick 2003). Pre-grown bacterial pellets were prepared according to Penrose and Glick (2003) and were inoculated on DF media plates and incubated for 3 days at 28ºC. Growth was monitored and colonies showing growth on ACC plates were taken as positive.

3.5 Estimation of salt-tolerance traits of isolates

Prior to the estimation of salt-tolerance traits of isolates, homogenous bacterial suspension were prepared by inoculating log phase culture (24 h) with OD 610 adjusted to 0.1 (CFU 10^8 cells/ ml) to specific media (according to the protocols). The culture filtrate was obtained by centrifugation at 10,000 rpm (*13,081 x g*) (21ºC) and further filtered through 0.45 µm membrane-filter (Merck, India) (Khare et al. 2011).

3.5.1 *Estimation of intracellular sodium ion accumulation*

The intracellular sodium ion level in bacterial biomass was estimated according to the protocol of Giri et al. (2013). The bacterial culture was inoculated in NB containing various amounts of NaCl (2-8%) and non-salinized control. The sample was incubated for 48 h at 25 ± 2ºC and after incubation cell biomass was collected by centrifugation (at *3000 x g*). The supernatant was discarded and the cell pellets were washed thrice with isotonic $MgCl_2$ solution (10 mM) and lysed by 30% HNO_3. Intracellular Na^+ was

measured using flame photometer and the amount was calculated using the standard curve of known sodium solutions (Systronics® flame photometer 130).

3.5.2 *Endogenous accumulation of osmoprotectant proline*

Freshly grown bacterial culture in NB (amended with NaCl and non-salinized control) was homogenized with 2 ml of sulphosalicylic acid (0.01 g/0.5 ml) and centrifuged at *20,000 x g* for 10 min. Subsequently, 1 ml of the homogenized supernatant was mixed with 1 ml acid ninhydrin reagent (prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and then 20 ml of 6M phosphoric acid was added and agitated until dissolved) and 1 ml glacial acetic acid was added and heated at 100°C in water bath for 1 h. Finally, the reaction was terminated in ice-bath. The cooled mixture was then extracted with 2 ml toluene, mixed vigorously and incubated for 30 min at room temperature. With the separation of two phases, the chromophore containing phase was warmed at room temperature and the optical density was measured at 520 nm using toluene as blank. The amount was estimated from standard curve using L-proline (Sisco-CHR, extrapure, 99 %). The amount of proline content was expressed as µg per mg of protein (the protein content of the culture was priorly detected using standard method of Bradford (1976)) (Bates et al. 1973).

3.5.3 *Reduction potential of isolates*

Ferric reduction potential of the isolates at various saline conditions was checked according to Xing et al. (2015). The supernatant (0.5 ml) of bacterial culture was mixed with 0.5 ml of 1% potassium ferricyanide solution and 0.5 ml phosphate buffer saline (pH 6.6). The mixture was then heated at 50°C for 20 min and allowed to cool. After cooling, 0.5 ml of trichloroacetic acid (TCA) (10%) was added to the mixture and then centrifuged at *3000 x g* for 5 min. The upper layer was mixed with 1 ml ferric chloride

solution (0.1%) and left for 1 min. The absorbance was measured at 700 nm and the standard curve was prepared using various concentrations of quercitin.

3.5.4 ***Antioxidant activity as per 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay***

The antioxidant activity exhibited by bacteria was estimated on the basis of reduction of DPPH, a stable free radical. The experiment was conducted using the protocol of Xing et al. (2015). The cell free extracts of freshly-grown culture (under various saline conditions) were obtained by centrifugation at 10,000 rpm (*13,081 x g*) (for 5 min at 4 °C). Subsequently, 1 ml of the supernatant was added to 1 ml of 0.2 mM DPPH solution (in methanol). The mixture was shaken vigorously and left to react for 30 min in the dark at room temperature. Deionized water was used as control. Absorbance was measured at 517 nm using Thermo Scientific™ Evolution 201 UV–Vis spectrophotometer and radical scavenging activity was calculated using the formula:

DPPH activity (% of inhibition) = [(A control − A sample)/ A control] × 100 ***-eq (1)***

where 'A control' is the absorbance of the control, 'A sample' is the absorbance of sample

3.5.5 ***Hydrogen peroxide scavenging activity***

The hydrogen peroxide (H_2O_2) radical scavenging activity exhibited by bacteria was determined by modifying the protocol of Ngonda (2013). Solution of 40 mM H_2O_2 was prepared in phosphate buffer (pH 7.4). Bacterial supernatant (1 ml) (grown under various saline conditions 2-8% NaCl and non-saline control) was added to H_2O_2 (5 ml) and incubated for 1 h at 37°C. Absorbance was measured at 560 nm against phosphate buffer as blank and percent of scavenging activity was calculated using the formula:

% hydroxyl radical scavenged = [Absorbance (control) − Absorbance (sample)/ Absorbance control] × 100 ***-eq (2)***

3.5.6 *Biofilming ability*

The biofilm formation by isolates under different saline conditions was observed using the protocol of Mathur et al. (2006). A loopfull of freshly grown bacterial culture was inoculated to test-tubes containing 10 ml of NB amended with various salt-levels (2-8% NaCl) and non-salinized control. The tubes were incubated for 48 h at 25 ± 2°C and 95 rpm. After incubation the culture medium with bacteria was discarded and tubes were washed with 3 ml of 1X phosphate buffer saline (PBS, pH 7). Subsequently, the tubes were flooded with 3 ml of 2% crystal violet solution and left for 15 min. The tubes were then washed with autoclaved distilled water and allowed to dry. The presence of biofilm rings on the inner wall of test-tube were visualized. For quantitative analysis, 1.5 ml of glacial acetic acid was added and optical density was measured at 570 nm.

3.5.7 *Biofilm formation on glass surface and visualization by Inverted Laser Scanning Confocal Microscopy (ILSCM)*

The detection of biofilm formation on glass surface under various saline conditions was done ILSCM. Confining to the objective of the study, to select salt-tolerant fluorescent pseudomonads, PE3 was only chekced for biofilming activity through ILSCM and SEM analysis. Freshly grown bacterial culture (taken in the ratio 2 ml culture and 2 ml NB containing various concentrations of salts: 0-8% NaCl) was kept in 6 well tissue culture plate (surface area 9.6 cm^2). Sterilized glass coverslip (20 mm) was then placed in each well and the plate was incubated at 28°C for 48-72 hours under static conditions. The coverslip was then removed from the incubated plates and was washed three times with 1X phosphate buffer saline (pH 7.2) to remove loosely attached cells from the glass surface (Ansari and Ahmad 2018). The coverslip was then stained with 0.1% acridine orange dye for 20 min and were examined for biofilm by ILSCM (ZEISS, LSM 900),

conducted at University Sophisticated Instrumentation Centre (USIC), BBAU, Lucknow. The depth or thickness of biofilm on the glass surface was analyzed by Z-stack analysis.

3.5.8 ***Development of biofilm on root surface and analysis through SEM and ILSCM***

Biofilm formation on root surface of sunflower plants was checked through SEM analysis and ILSCM. Surface sterilized sunflower seeds were grown aseptically in water agar upto two to three leaf stage. The seedlings were then uprooted and washed with sterilized distilled water for 3-4 times. After washing, roots of the plants were dipped in overnight culture of bacteria (CFU ~ 10^5 per ml) and incubated at 28^{o}C for 48 hours. Additionally, to determine the role of EPS in biofilming and colonization of bacteria, extracted and purified EPS (2% w/v) was added to culture broth at the time of dipping the roots. Only EPS added to broth (2% w/v) was used to study the difference and untreated roots were taken as negative control. Subsequently, the seedlings were washed 3-4 times with sterilized distilled water to remove loosely adhered cells from the root surface (Ansari and Ahmad 2018).

For SEM analysis the root samples were fixed in 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 4 hours and were dehydrated using gradients of ethanol series (30-100% v/v) and critical point dried (CPD) in carbon dioxide were used (Smith et al. 1990). The samples were subsequently viewed under SEM (Model: JEOL, JSM6490LV).

To analyze the root samples for confocal laser microscopy the roots (treated with bacteria and bacteria plus EPS) were stained with acridine orange for 10 mins and the longitudinal sections of the roots were placed on sterile glass slide and covered with coverslip. The roots were then visualized by ILSCM (ZEISS, LSM 900) at five randomly selected positions for biofilm formation.

3.6 Identification of bacteria

The selected isolates were identified through molecular analysis (16S rRNA sequencing) and proteomic approaches (matrix-assisted laser desorption ionization time of fight mass spectrometry (MALDI-TOF-MS)).

3.6.1 *Proteomic analysis through MALDI-TOF MS Biotyping*

Identification of the isolates were done by MALDI-TOF MS Biotyping at National Centre for Microbial Resources (NCMR), Pune, India. Actively grown culture was spotted on MALDI target plate (Bruker Daltonik GmbH, Germany) and developed as thin film. The bacterial smear was then overlaid with 1 µl of HCCA (α-Cyano-4-hydroxycinnamic acid) solution and allowed to dry at room temperature. Bacterial standard (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin, supplied by Bruker Daltonik GmbH, Germany) (1 µl) was loaded to different well and 1µl of HCCA matrix to it. Finally, the dried targeted plate was subjected to the instrument AUTOFLEX speed (Bruker Daltonik GmbH, Germany). For analysis on MALDI-TOF MS instrument, mass spectra were detected in linear positive ion extraction mode at a laser frequency of 200 Hz within a mass range from 2,000 to 20,000 Da (ion source 1 voltage was 19.5 kV, ion source 2 voltages were maintained at 18.2 kV, lens voltage at 7kV) and the extraction delay time was 240 ns. The visualization of bacteria and mass spectra were done using MALDI biotyper software 3.0 (Bruker Daltonik GmbH, Germany) equipped with MBT 6903 MPS Library (released in April 2016). The obtained log value if is ≥ 1.7 then the strains are confirmed as the member of that genus and if log value is ≥ 2.0 are confirmed as the member of that species (Rahi et al. 2016).

3.6.2 *Molecular analysis through 16S rRNA sequencing*

The isolates were grown on NA plates at 25 ± 2°C for 24 hours and DNA was extracted using standard protocols. Molecular identification of bacteria was done by sequencing of 1.5 kb 16S rRNA sequences using the universal primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5′-GGTTACCTTGTTACGACTT-3. Conditions for amplification reaction was maintained as following:

PCR conditions:

Reaction Mixture (50 µl)		**Cycling Conditions**		
Template DNA	100 ng	Initial Denaturation	2 minutes at 95°C	35 Cycles
Forward Primer	0.3 µM	Denaturation	30 seconds at 95°C	
Reverse Primer	0.3 µM	Annealing	30 seconds at 52°C	
Master Mix	25 µl	Extension	2 minutes at 72°C	
Nuclease Free Water	Volume makeup 50 µl	Final Extension	15 minutes at 72°C	

Primer details for PCR:

S. No	**Oligo name**	**Sequence (5'-3')**	**Tm (°C)**	**GC-content**
1	27F	AGAGTTTGATCMTGGCTCAG	56.3	47.5%
2	1492R	CGGTTACCTTGTTACGACTT	55.3	45%

Primer details for sequencing:

On the basis of PCR results, single amplified fragment was used for sequencing of 16S rRNA gene from both the strands and the primers used are discussed below:

S. No	**Oligo name**	**Sequence (5'-3')**	**Tm (°C)**	**GC-content**
1	785F	GGATTAGATACCCTGGTA	56.3	47.5%
2	907R	CCGTCAATTCMTTTRAGTTT	55.3	45%

The resulting amplicons (5 µl) of the 16S rRNA genes were analyzed by gel electrophoresis and were mixed with loading buffer (2 µl). The product was then electrophoretically examined through 1% agarose gel stained with ethidium bromide (10 mg/ ml) at 100 V for 60 min. The bands were observed under UV light and image was captured.

3.6.3 ***Phylogenetic tree analysis, sequence submission to National Center for Biotechnology Information-(NCBI) GenBank and strain submission to culture collection center:***

The 16S rRNA sequences of the isolates were then run on NCBI-Basic Local Alignment Search Tool (BLASTn) programme (Altschul et al. 1990) and Ez-Taxon server (Yoon et al. 2017) and submitted in GenBank for accession number. Phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei 1987) in MEGA X software (6.0 version) (Kumar et al. 2018). The selected potential isolates were also deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, India, an international culture repository.

3.7 Characterization of exopolysaccharide under different saline conditions

The extracted EPS as mentioned in section 3.4.8, both produced under saline and non-saline conditions were purified (through dialysis) and the effect of salinity on properties and composition of the macromolecule were determined.

3.7.1 ***Compositional analysis***

(a) Colorimetric analysis

(i) Carbohydrate content

The carbohydrate content of purified EPS extracted at various saline conditions was estimated using phenol-sulphuric acid method according to Dubois et al. (1956). The

aqueous solution of EPS (2% w/v) (200 µl) was mixed with 600 µl of conc. H_2SO_4. The mixture was then vortexed for 10 min followed by addition of 120 µl phenol (5% aqueous solution; pH 5.6). The mixture was heated at 90°C for 5 min. Cooling the tubes to room temperature, the absorption was measured at 490 nm and amount of carbohydrate was estimated from the standard curve of D-glucose (Sigma-Anhydrous).

(ii) Protein content

The protein content of EPS was checked according to the protocol of Gong et al. (2009) modifying from the standard method of Lowry et al. (1951). The aqueous solution of EPS (2% w/v) was prepared for the test and 0.3 ml was re-suspended in 10 ml distilled water. To the solution 1.5 ml alkaline copper reagent (prepared by adding 1 ml of 2% sodium-potassium tartarate, 1 ml of 1% $CuSO_4.5H_2O$ to 98 ml of 2% sodium carbonate in 10^{-1}M NaOH) was added followed by addition of 75 µl Folin and Ciocalteu's phenol reagent. The mixture was incubated for 30 min at room temperature and the optical density was measured at 500 nm. The amount of protein expressed as mg per gram of dried EPS was calculated from standard curve of bovine serum albumin (BSA, Sisco-pH 6–7, fraction V) (BSA, 1 mg/ ml).

(iii) Phenol content

Total phenolic content of EPS was detected using the protocol of Seo et al. (2015). The aqueous suspension of EPS was prepared (100 µg/ ml) and 50 µl aliquot was mixed with 50 µl of 5% Folin-Ciocalteu's reagent. The tubes were incubated at room temperature for 5 min in the dark and then 100 µl of 20% sodium carbonate solution was added. Again after incubating for 20 min at room temperature the development of blue color was observed and the observance was measured at 730 nm. Total phenolic content was expressed as gallic acid equivalents (GAE) from the standard calibration curve of gallic acid (conc. 50-250 µg/ ml).

(iv) Detection of reducing sugars

Reducing sugars were traced in EPS as per Benedict's method (1907) using Fehling's solution. Fehling A solution: prepared by dissolving 7 g $CuSO_4.7H_2O$ in distilled water and making the volume to 100 ml; Fehling B solution: prepared by dissolving 24 g KOH and 6g sodium-potassium tartarate in distilled water and the volume made upto 100 ml. The two solutions (A and B) were mixed in the ratio 1:1. Subsequently, 5 ml of Fehling's solution was mixed with 0.2 ml of EPS suspension (2% w/v) and the tubes were boiled for 2-3 minutes. The change in color was noted and the interpretation according to color-change was as per following:

Change in color	**Estimation of reducing sugars**
No change in color (blue)	Absence of reducing sugars
Blue to green	0.1 to 0.5 g of reducing sugars
Blue to yellow	0.5 to 1.0 g of reducing sugars
Blue to orange	1.0 to 2.0 g of reducing sugars
Blue to brick red	Over 2 g of reducing sugars

(b) ***Detection of functional groups and monomeric unit of EPS***

(i) Fourier Transformed-Infrared (FT-IR) spectroscopy

The extracted EPS both under salt-stressed and non-stressed conditions were completely dried and mixed with spectral grade potassium bromide (KBr). The pellets were checked through FT-IR (Thermo-Scientific (Nicole 6700)) at USIC, BBAU, Lucknow, in the range 4000−400 cm^{-1} (Sardari et al. 2017). The difference in the presence/ absence of functional groups upon salt exposure was examined.

(ii) Gas-chromatography mass spectrometry (GC-MS) analysis

The monosaccharide composition of EPS was determined by GC-MS analysis. Hydrolysis and silylation of dried and powered EPS was carried out to obtain volatile derivatives (methylsilanes) and then was subjected to GC-MS analysis. To obtain the

derivatives, 5–10 mg of EPS was hydrolyzed with Trifuoroacetic acid (1M) at 100°C for 8 h followed by reduction with 100 µL $NaBH_4$ (10 mg in 1 mL of 1M ammonium hydroxide) for 16 h at room temperature. The samples were then silylated with N, O-Bis (trimethylsilyl) trifuoroacetamide (BSTFA) in pyridine at 60°C for 30 min (Yoshiba et al 2015; Kumar et al. 2019). The GC-MS analysis of silylated EPS in pyridine was conducted using Shimadzu GC-MS-QP 2010 Plus instrument equipped with a capillary column Rtx-5 (dimensions: 0.25-µm film thickness, 0.25mm ID, 30m in length) and conditions were standardized according to Yoshiba et al. (2015). The data were then matched with National Institute of Standards and Technology (NIST) library (DOI: https://doi.org/10.18434/T4D303) to find the related compounds.

(c) Determining the structure and crystallinity through XRD

The detection of EPS composition, crystallinity (i.e. amorphous or crystalline) under various saline conditions in comparison to non-saline control was analyzed through X-ray diffraction (XRD) conducted at Birbal Sahni Institute of Paleobotany, Lucknow, India, and USIC, BBAU, Lucknow, India. PANalytical X′Pert Pro Powder X′Celerator diffractometer instrument was used and the range (of 2θ) between 20° and 80°. Crystallinity index was calculated using Match software (version 3.11). Material structure was determined by measuring the spacing between the layers of atom or planes (d_{hkl}) using the same software (Dogan et al. 2015).

(d) Structure morphology and elemental composition through SEM and EDS

The morphology and microstructure of dried purified EPS was determined through scanning electron microscopy (SEM) (Model: JEOL, JSM6490LV) at USIC, BBAU, Lucknow. Further, the elemental composition of EPS obtained at different saline conditions was determined by Energy Dispersive Spectroscopy (EDS) (EDS 133, EV Dry Detector (INCA x-act) of OXFORD instruments, UK) at USIC, BBAU, Lucknow.

The difference in composition and morphology of EPS according to salinity was examined.

3.7.2 *Detection of salt-tolerance traits of EPS*

The extracts of purified EPS were prepared in various solvents including water, methanol, and phosphate-buffer for examining the salt tolerance traits (concentration of extract according to specific protocols).

(i) Reduction potential

The reducing power of EPS (produced at saline and non-saline conditions) was checked according to Xing et al. (2015). The aqueous extract of EPS was prepared (100 µg/ ml) and 0.5 ml of sample (EPS extract) was briefly mixed with equal amounts of 1% potassium ferricyanide and phosphate buffer-saline (pH 6.6). The resultant mixture was then heated at 50°C for 20 min and upon cooling 0.5 ml of 10% trichloroacetic acid (TCA) was added and mixed vigorously. To 1 ml of the upper layer, ferric chloride (0.1%, 1 ml) was added and made to react for 10 min. The absorbance was measured at 700 nm and standard curve was drawn using quercitin (Hi-media, HPLC grade) in the range of 0.1− 0.8 mg/ ml.

(ii) Antioxidant activity

The ability of EPS to scavenge ROS was determined as the function of inhibiting DPPH, a stable radical. DPPH solution (0.2mM) and EPS extract (100 µg/ ml) were prepared in methanol. Freshly prepared DPPH solution and EPS extract were mixed together in 1:1 ratio, mixed vigorously and left for incubation in the dark for 30 min at room temperature. Deionized water instead of EPS was used as control and absorbance was measured at 517 nm (Xing et al. 2015). The radical scavenging activity of the sample was measured using the formula discussed in equation **eq 1** (mentioned in section 3.5.4).

(iii) Hydroxyl scavenging activity

ROS scavenging potential of EPS was determined (ROS here: superoxide radical O_2^-) following the protocol of Ngonda (2013). The solution of H_2O_2 (40mM) prepared in phosphate buffer (pH 7.4) and the extract of EPS (conc, 100 µg/ ml) in the same solvent were mixed (both 1 ml). After 30 min of incubation, absorbance was measured at 560 nm. The scavenging activity was measured using the formula mentioned in equation **eq 2** (mentioned in section 3.5.5).

(iv) Flocculation activity

To check the ability of soil aggregate formation, flocculation activity of EPS was monitored using saline soil using the protocol by Deng et al. (2003). EPS was mixed in distilled water (1 ml; 50 mg/ L); in separate tubes 5 ml of saline soil slurry was prepared (conc. 0.04 g/ ml) and vortexed thoroughly until mixed. Subsequently, EPS extract was added to this soil mixture and homogenized. After leaving the homogenized mixture for 10 min the optical density was measured at 550 nm and flocculation activity was calculated using the formula:

Flocculation property% = [(B-A)/ B] x 100

where A is optical density of the sample (EPS) plus soil and B is optical density of only soil.

(v) Emulsification activity

To determine the emulsification activity of EPS, the standard protocol of Cooper and Goldenberg (1987) was followed. EPS was dissolved (10.5 % w/v) in 5 mL distilled water and mixed in test tubes along with addition of 5 mL hydrophobic substance (olive oil) and then vortexed thoroughly for 2 min.

After leaving the mixture for 24 h at 25ºC, the percent of emulsification index was calculated using the formula:

Emulsification index (%) = [(Height of emulsion formed)/ (Total height of solution)] x 100

(vi) Accumulation of sodium ions

The aqueous solution of EPS was prepared (at conc. 10 mg/ ml) and taken in dialysis bag (12–14 kDa MW cut-off, Hi-Media Laboratories Pvt. Ltd., India) (4 ml). The dialysis bag containing EPS solution was then transferred to beaker containing 80 ml of 8% (w/v) NaCl solution (any saline solution 5, 10, 20% can be taken). The beaker was then placed on magnetic-stirrer overnight at 28 ± 2ºC. After completion of the process, the process of dialysis was repeated 2-3 times using distilled water instead of saline solution to remove excess of unbound NaCl. Subsequently, the solution containing EPS and bound Na^+ was checked for sodium accumulation using flame photometer (Systronics® flame photometer 130). The standard was prepared using known samples of sodium and distilled water (instead of EPS) was taken as negative control (Mukherjee et al. 2019).

3.8 Effect of EPS and selected isolates on sunflower growth, yield and salt-tolerance traits

3.8.1 Preparation of bioformulation

For the preparation of talc-based bioformulation protocol of Nandakumar et al. (2001) was followed. To 1 kg talc, 15 g calcium carbonate ($CaCO_3$) was added to adjust the pH to neutral. Further, 10 g carboxymethyl cellulose (CMC) was added to the mixture (talc-$CaCO_3$) to enhance the adhesion property of the bioformulation and subsequently the talc-cellulose mixture was autoclaved. For preparing bioformulation, selected isolates were grown in NB for 24 h at 25 ± 2ºC and 120 rpm shaking and the optical

density was adjusted to ~ 1 (at 610 nm) (corresponding to $3.0 \pm 0.5 \times 10^8$ CFU/ ml) after incubation. This bacterial suspension (450 mL) was added to autoclaved talc-cellulose mixture and left overnight to adjust the moisture content to ~35 %. However, for only EPS treatment, this step was skipped with no addition of bacteria to talc-mixture. The talc-suspension (in autoclaved distilled water) was prepared at the rate of 20 g/ L (as per Nandakumar et al. 2001). Confining to the objectives of the study (application of fluorescent pseudomonads), EPS extracted from PE3 was selected and characterization of AF7-EPS is ongoing; however, for bacteria and metabolite combination treatments, respective EPS was used to ensure compatibility. Due to excellent PGP and salt-tolerance properties exhibited by AF7, the strain was considered in the study. To the suspension, purified-dialyzed EPS (selected EPS with most significant properties) was added at various concentrations (0.1 %, 1.0 %, 2.0 %, 5.0 % w/v) according to the respective treatments. The combination of bacteria and metabolite was prepared by diluting 20 gm talc-bioformulation (with added bacteria) in 1000 mL autoclaved-distilled water and then adding 20 mg of EPS produced by that particular bacteria to the suspension (bacterial population: 10^7 CFU/ ml talc suspension). The treatments designed were: **T1- 0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4- 5.0% EPS, T5- 2% EPS+PE3, T6- 2% EPS+AF7, T7- AF7 only, T8- PE3 only, T9-untreated control**. (where, AF7 and PE3 are selected bacterial isolates).

(a) *In vitro* study

Pot experiment was conducted in triplicates in earthen pots sized 24×12×12 cm during the months of February-May for two consecutive years (2018 and 2019). Saline soil was collected from adjoining areas of Lucknow, Uttar Pradesh, India (26° 72 E, 80° 85 N). The temperature during the pot study was: maximum 48° C and minimum 9° C. The pots were filled with 8 kg autoclaved saline soil without addition of fertilizers. The

soil used for pot trial had EC 9.2 dS/m, pH 8.1, organic carbon content 3.3 gm/ kg, available nitrogen 0.15 gm/kg, available phosphorous 41.3 mg/kg, available potassium 156 mg/kg, soil organic matter 5.7 gm/kg and microbial biomass 103.5 mg/kg (content estimated according to Jackson (1973) and Zhang et al. (2019b)). Prior to sowing, *H. annuus* (sunflower variety A-H-305) seeds were surface sterilized with 3% hydrogen peroxide solution (for 5 min) and then washed 5-6 times with autoclaved distilled water (Bakhsh et al. 2016).

The sterilized sunflower seeds were soaked in prepared bioformulation suspension and left overnight for proper coating. After soaking, the seeds were dried for 2–3 h to adjust moisture content to ~ 35%. The seeds were then sowed in pots (5 seeds per pot) and thinning was performed after germination to one seed per pot for homogeneity. The germination rate (per pot) was calculated using the formula (Kader 2005):

Germination % = ((Number of germinated seeds/ total number of seeds) × 100)

-eq (3)

Root colonization of isolates was checked (for treatments T5, T6, T7, T8) at various plant growth stages (30, 60, 90 and 120 days after sowing (DAS)) (Cavaglieri et al. 2009). The roots of randomly picked plants were washed and weighed and then blended and re-suspended in phosphate-buffer saline (PBS). The suspension was then serially diluted and spread on selective media of the isolates (Pseudomonas agar media (for fluorescein) for *Pseudomonas* (Hi-media)) and CFU was calculated per gram of root. After comparing the morphology of the bacteria obtained on plates, with AF7 or PE3, randomly picked colonies were identified through 16S rRNA sequencing and counted (Tewari and Sharma 2020).

(b) ***In vivo study***

The field trial was done consecutively for two years in Ambedkar Nagar, 26◦46 E, 82◦57 N, Uttar Pradesh, India, during February to May (2018 and 2019). The temperature reported during the time of field trial was 42ºC (maximum) and 12ºC (minimum). At the time of sowing the maximum temperature was 24ºC and minimum 12ºC. The soil of the experimental field was alkaline (pH 11.9) with EC of 15.9 dS/m, categorizing it as highly saline. The nutrient status of the soil was: 3.26 g/ kg organic carbon, 0.16 g/ kg available nitrogen, 41 mg/ kg available phosphorous, 158 mg/ kg available potassium, 5.6 g/kg organic matter, and 110.35 mg/kg microbial biomass. The experimental set up was in randomized block design (RBD) and the size of each block was 3×3 m^2. The distance between two plants was ~30 cm and between two rows was ~60 cm (50 seeds in each block), and each treatment was conducted in triplicate. The germination rate was calculated per block (using equation **eq 3,** mentioned in section 3.8.1 (a)).

3.8.2 Analysis of growth parameters of sunflower plants

The plants were uprooted 120 days after sowing (DAS), rinsed with water and checked for various growth parameters including fresh weight, dry weight, root length, shoot length, stem-girth, head size of flower, seeds per plant, total weight of seeds per plant and seed yield. The oil content from the seeds were extracted using Soxhlet apparatus, analytical-reagent grade n-hexane as solvent (90 %) and at 60–72ºC for 8 h (Baümler et al. 2016).

3.8.3 Analysis of physicochemical and biochemical properties of sunflower plants

Further for physicochemical analysis of plant leaves, the extracts were prepared in various solvents including methanol, ethanol, acetone, phosphate buffer (0.1 M, 7.6 pH) and distilled water at the concentration of 50 g/ 500 mL. The filtrate was collected by

centrifugation and the desired extract was stored for further use (Das et al. 2014). The qualitative analysis of various plant components including tannins, saponins, polyphenols and steroids were done according to Sylvie et al. (2014). The biochemical properties were checked using following protocols:

(i) Chlorophyll content

Chlorophyll content was measured by standard protocols according to Arnon (1949). The leaf extract was prepared in acetone (10mg/ ml) mixed well and kept at 4°C in dark for overnight. After this period the supernatant was collected through centrifugation and the absorbance was measured at 663 and 645 nm. The amount of total chlorophyll was obtained using the formula:

Total chlorophyll = 20.2 (A_{645}) + 8.02 (A_{663})

where **A_{645}** is absorbance at 645 nm and **A_{663}** is absorbance at 663)

(ii) Carotenoid content

Carotenoid content of the leaf extract (in acetone) was checked according to Harborne (1973). The absorbance was measured at 480 nm and the amount of carotenoid was calculated using the formula:

Amount of carotenoid (mg/ gm) = (4 x Abs_{480} x total volume of sample)/ weight of fresh plant tissue

(iii) Total flavonoid content

The quantitative analysis of flavonoid content of leaves (aqueous extract) was determined by aluminium chloride colorimetric method according to Shubhangi et al. (2017) using quercitin (Hi-media, HPLC grade) solution in methanol as standard.

(iv) Phenolic content

Phenolic content of leaves (ethanolic extract) was determined according to Lee et al. (2015) using Folin-Ciocalteu reagent and the absorbance was read at 765 nm using gallic acid ((Hi-AR™/ACS) as standard.

(v) Carbohydrate content

The sugar content of the leaves was estimated through phenol-sulphuric acid method using 5% phenol and 96% sulphuric acid (reagent grade), taking D-glucose (Sigma-Anhydrous) as standard (Dubois et al. 1956).

(vi) Protein content

Protein content of leaves was checked according to the standard method of Lowry et al. (1951) using BSA (Sisco-pH 6–7, fraction V) as standard.

3.8.4 Analysis of salt-tolerance traits of sunflower plants

(i) Accumulation of osmoprotectant proline

The proline content of leaves was estimated according to the standard method of Bates et al. (1973) using acid-ninhydrin reagent. Firstly, the leaf sample (0.5g) was homogenized with 2 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation (*12000 x g* for 10 min). Now, homogenized leaf extract was mixed with acid-ninhydrin reagent and glacial acetic acid in the ratio 1:1:1. This suspension was then heated at 100^{o}C for one hour and after cooling to room temperature, was extracted with 2 ml toluene and shaken vigorously. After 30 min of incubation, the separation of chromophore was observed and the 1 ml of upper layer was checked for optical density at 520 nm. The standard was drawn using L-Proline ((Sisco-CHR, extrapure, 99 %) as standard.

(ii) Reduction capacity

The aqueous extract of leaf (500 µg/ ml) was mixed with 0.2 M phosphate buffer (pH 6.6). The mixture was then mixed with freshly prepared potassium ferricyanide (2% w/v) and incubated in water bath at 50°C for 20 min. Then, 1 ml of 10% TCA was added and the mixture was centrifuged at *1177 x g* (3000 rpm) for 10 min. The obtained supernatant (2 ml) was mixed with 2 ml distilled water and 500 µl ferric chloride (1%). Absorbance was measured at 700 nm (Senguttuvan et al. 2014).

(iii) Antioxidant activity

The antioxidant activity of leaf (methanolic extract) was measured according to Xing et al. (2015). The absorbance was measured at 517 nm and the activity was determined as percent of DPPH inhibition.

(iv) Hydroxyl scavenging activity

The hydroxyl scavenging activity of leaf was determined according to the protocol of Ngonda (2013) using leaf extract in phosphate buffer.

(v) Relative water content (RWC)

RWC of the leaves was determined using the protocol of Weatherley (1950). Initially the fresh weight of leaf (of each treatment) was recorded. The leaf was then dipped in distilled water (equal amount in each tube) and left for 24 h in refrigerator. The turgid leaf weight was now recorded and next the leaf was dried in oven at 70°C for 72 h. The dry weight of the leaf was measured and RWC was calculated using the formula:

RWC= [(Fresh mass – Dry mass)/ (Turgid mass – Dry mass)] x 100

(vi) Electrolyte leakage (EL) and membrane stability

The leaves of the plants were uniformly cut into discs with the help of paper puncher and transferred to test tubes containing 25 ml distilled water. The tubes were then

vortexed for 10 s and the initial EC was measured (EC_0). The tubes were now placed in refrigerator for overnight and then EC was measured (EC_1) (Yang et al. 1996). The test tubes were then autoclaved at 121°C for 20 min and EC was recorded (EC_2).

EL was calculated using the formula:

EL (%) = [(EC_1 - EC_0) / (EC_2 - EC_0)] x 100

Membrane stability index (MSI) was calculated using the formula:

MSI (%) = [(EC_2 - EC_1) / EC_2] x 100

3.9 Statistical analysis of results and correlative analysis of various treatments

The statistical analysis of the data was done using MS Excel, XLSTAT 2020 (Addinsoft 2020), and IBM SPSS Statistics 20, R-studio (version 3.6.2). The relation between the variables was detected using Pearson correlation coefficient, the model significance was predicted using regression analysis. The correlation matrix based on Pearson method and matrix for coefficient of determination were drawn using XLSTAT to analyze the response model of bacteria against salinity.

Analysis of variance (ANOVA) was conducted using IBM SPSS to compare the significant or insignificant difference in effect of treatments on sunflower plants using Duncan's Multiple Range test (DMRT) with least significant difference at 5% level of significance ($\alpha = 0.05$). Heatmap based on Pearson and Ward for determining distance and clustering, was drawn using R-studio to visualize the difference between various treatments and salt-tolerance, growth responses of sunflower ($P < 0.05$) and also to conclude the most potential treatment. Correlogram based on Pearson correlation analysis ($P < 0.05$) was constructed using R-studio to prove the relationship between EPS inoculation and salt tolerance-properties of treated sunflower plants.

RESULTS

4.1 Isolation of salt-tolerant bacteria

Soil of various sampling sites **(Fig. 8)** was analyzed and the salinity, pH and nutrient status are mentioned in **Table 2**. The soils were mostly saline and as per the extent of salinization some sites (K2 and K3) were highly saline with EC>16 dS/m. High pH of soil (pH > 8.0) also characterized the soils as alkaline or showing effect of both salinity and sodicity. In total 42 bacterial isolates were obtained from saline soil, of which 12 were selected based on salt-tolerance (**Fig. 9**). The screened isolates showed tolerance up to 8% NaCl conditions (or 1400 mM NaCl). Seven of twelve isolates showed fluorescence under UV light **(Fig. 9)**.

4.2 Phenotypic characterization

The colonies of all the isolates were convex (except T1K1) **(Fig 9).** Except for highly pigmented isolates (AF2, AF7, AF6, SS2, P1B, T1K1) all the isolates showed smooth margins. T1K1 formed dry colonies with rough margins and were dark-brown colored. About 90% isolates were Gram negative and remaining 10% were Gram positive **(Fig. 10).** All the isolates were rod shaped and 76% of the total isolates were motile while 24% were non-motile **(Fig. 11; Table 3**). Of the total bacterial isolates (42), colonies of 88% isolates were mucilaginous while 12% were non-mucilaginous. It was observed that in case of selected isolates (12) when salinity was increasing the colonies became more mucilaginous. Most of the bacterial isolates were fast growing with generation time 2-4.5 h and few were slow growers (generation time above 6 h). About 81% of isolates showed fluorescence under UV light. The isolates with the ability to tolerate 8% NaCl were selected for further studies (12 isolates).

4.3 Biochemical and physiological properties

Apart from examining the salt-tolerance in bacteria, the growth pattern under different pH and temperature conditions were also checked. All the isolates grew at pH 6 to 10

and temperature 15, 25 and 35°C. Only 8 isolates could grow under highly acidic conditions (pH 4) **(Table 4).** At lower temperature (5°C) only 2 isolates were able to grow and similar was the condition at high temperatures (45°C) where only 5 isolates showed growth **(Table 4).**

The salinity growth curve was drawn for the selected isolates (AF7 and PE3) showing highest salt-tolerance and maximum PGP properties. Isolates PE3 and AF7 were evaluated for growth and survival at various salt levels and was found to be tolerant up to 8% NaCl. PE3 and AF7 growth was not affected upto 2% NaCl, however, salinity above 4% NaCl significantly reduced the growth rate. Mean doubling time under non-saline conditions was found to be 270 min (for PE3) and 240 min (for AF7) which increased to 275 min (PE3) and 246 min (AF7) at 2% NaCl. Maximum doubling time of 743 min and 613 min was reported at 8% NaCl for PE3 and AF7 respectively **(Fig. 12).**

As far as utilization of carbon sources was concerned, it was found that majority of the isolates (83%) were able to grow on glucose and the next preferred carbon source were mannitol (74%), sucrose (71%), glycerol (69%) and galactose (67%) **(Table 5).** Only one isolate (PE3) was able to utilize malate as carbon source. All the isolates were able to utilize yeast extract, KNO_3 (except T1K6), $NaNO_3$, tryptophan and NH_4Cl as nitrogen source in the medium. The least preferred nitrogen source were glycine and methionine, with merely 14% and 17% isolates growing on the amended medium with these two nitrogen sources respectively **(Table 6).**

All the isolates showed ammonia production and except two (P1B and KS1) all the other isolates were also positive for catalase activity (**Table 7**). Majority of the isolates (values/ 42) were positive for amylase test (39), PHB accumulation (35), growth on

GPA (32), VP test (34) and citrate test (29). Only few bacteria were positive for protease (8), cellulase (7), H_2S production (4) and MR test (3) (**Fig. 11, Table 7**).

4.4 Plant growth promoting properties under various salt concentrations

All the isolates were able to produce EPS and nearly 83% were able to solubilize zinc and synthesize siderophore. Of the total, 74% were positive for GA production and 69% for IAA production. About 71% isolates showed phosphate solubilization and 69% produced HCN (important in biocontrol activity) **(Table 8).** However, comparing the quantitative analysis of various PGP traits, AF7 and PE3 showed highest amount for majority of the properties. Therefore, screening and selecting the isolates on the basis of salt-tolerance levels and PGP traits, AF7 and PE3 were selected for further characterization.

4.4.1 *Phosphate solubilization*

The phosphate chelation activity of PE3 and AF7 was found to be increasing from non-saline condition to maximum at 2% NaCl. PE3 (309.12 ± 3.82 µg) was a better phosphate solubilizer in comparison to AF7 (126.03 ± 0.1 µg). Above 2% NaCl, the property was absent in AF7 while PE3 was able to solubilize P upto 6% NaCl however, the amount of phosphate solubilized significantly reduced (**Table 9**, **Fig. 13**). Determining the relation between salinity and P solubilization, above 2% NaCl there was negative correlation found for both the strains (coefficient of correlation (r): -0.905 for PE3 and -0.846 for AF7) **(Fig 13, 14 and 15).** This inversely proportional ratio was supported by regression analysis with R^2 value of 0.818 and 0.799 for PE3 and AF7 respectively.

4.4.2 *Zinc solubilization*

Against the gradients of salinity, both PE3 and AF7 were able to chelate zinc upto 6% NaCl (**Fig. 13**). In case of PE3 there was about 28% increase in zinc solubilization

activity when salinity was increased to 2% NaCl from non-saline control; similarly in AF7 the activity also increased by 16% from non-saline condition to 2% NaCl. The property was significantly (as compared to non-saline control) maintained upto 4% NaCl and then declined and no solubilization (halo zone) was found at 8% salinity (**Table 9**). Interpreting from statistical analysis it was reported that salinity above 4% NaCl had negative impact on the trait and correlation coefficient (r) was equal to -0.820 and -0.790 corresponding to R^2 value of 0.672 and 0.596 for PE3 and AF7 respectively (**Fig. 14 and Fig. 15**).

4.4.3 *Potassium solubilization*

Isolate PE3 was able to chelate potassium from insoluble source (mica) however, the property was absent in AF7. Regarding the impact of salinity on K solubilization, the chelation activity was maximum at 2% NaCl (**Table 9**), increasing by about 14% in comparison to non-saline conditions. Salinity above 2% NaCl reduced the activity and no solubilization was found at 6% and above NaCl conditions (**Fig. 13**). The negative correlation of -0.905 (r value) and R^2 value of 0.815 was reported determining the model between salinity and K-chelation (**Fig. 14 and 15**).

4.4.4 *Siderophore production*

Siderophore production on CAS agar plates showed orange to yellow colored zone for both PE3 and AF7 inoculated plates. Quantitative analysis revealed that maximum siderophore production by PE3 was at 2% NaCl (74.81 ± 1.54 psu) and was maintained till 6% NaCl (64.74 ± 4.62 psu), but at 8% NaCl no siderophore production was reported (**Table 9**). The correlation coefficient (r) was found to be -0.774, the fitness of the model was not predicted as R^2 was equal to 0.599 (**Fig. 14 and 15**). AF7 was more efficient siderophore producer (95.28 ± 1.45 psu at non-saline condition) as compared to PE3. AF7 also managed to produce siderophore up to 6% NaCl, but higher salinity

inhibited it's production. Salinity had significant (negative) impact on siderophore production ability of AF7 and the correlation coefficient (r) was -0.961 and model was a better fit (R^2 value 0.921).

4.4.5 ***IAA production***

PE3 isolate was a potent IAA producer (as compared to other isolated cultures) and the strain was able to produce the phytohormone even at 8% NaCl (**Table 9**). Optimizing the salt-conditions for IAA production, at 2% NaCl, IAA production was maximum for both the strains (AF7 and PE3). The correlative analysis indicated positive relation (r) ~1 for IAA up to 2% NaCl (for AF7 and PE3). Observing from linear regression analysis (**Fig. 14 and 15**) for PE3, the negative slope with coefficient of determination R^2 = 0.655 for IAA with correlation coefficient (r) -0.809 established the model for their reverse relationship with salinity (especially above 2% NaCl). Similarly, for AF7 correlation coefficient (r) was equal to -0.749 with coefficient of determination R^2 = 0.543, showing lesser fit model for salinity-IAA relationship (**Fig. 13**, **14 and 15**).

4.4.6 ***GA production***

Comparing the GA production against salinity, it was found that in case of AF7 upon exposure to salinity, (from non-saline to 2% NaCl) there was an exponential increase in the phytohormone production (**Fig. 13, Table 9**). There was almost 118% increase in GA production when AF7 was exposed to salinity. Therefore, optimization study revealed that AF7 is a better phytohormone producer under salinity stress. The isolate (AF7) was able to produce GA even at 8% NaCl however, the quantity was highly reduced at highest saline condition. The correlative analysis indicated positive relation (r) = 0.75 for GA up to 4% NaCl for AF7. Linear regression analysis was unable to establish fitness of the model for salinity versus GA production (upto 8% NaCl, for

AF7) as correlation coefficient (r) was equal to -0.310 with R^2 value equal to 0.082 (**Fig. 14 and 15**).

GA production in PE3 was maximum at 2% NaCl however, there was lesser fold-increase in synthesis upon exposure to salinity as compared to AF7 (**Table 9**, **Fig. 13**). Relatively, there was 10% increase in GA production by PE3 from non-saline control to 2% NaCl. PE3 maintained phytohormone synthesis upto 8% NaCl but there was decrease reported above 2% NaCl and negative correlation (r) of -0.886 with good-fit model ($R^2 = 0.786$) (**Fig. 14 and 15**).

4.5.7 *HCN production*

Qualitative analysis of HCN production by PE3 and AF7 revealed that both the strains were able to produce HCN proving their candidature as biocontrol agents. PE3 produced HCN at all salt levels studied and most color change from yellow to red-brown was observed at 2% NaCl. However, in AF7 the production of HCN was reported only upto 2% NaCl and highest color change was under non-saline condition (**Fig. 13, Table 8**).

4.5.8 *Biocontrol activity*

Checking the biocontrol activity of the bacteria (42 isolates), isolate T1K1 was the most potent antagonist against all the fungal phytopathogens *i.e. M. phaseolina, F. solani, F. oxysporum, A. brassicae* inhibiting fungal growth by more than 50%. Among all the isolates, 76% were able to inhibit *M. phaseolina*, 67% restricted growth of *F. solani*, 83% inhibited *F. oxysporum* and 45% exhibited biocontrol activity against *A. brassicae* (**Table 11, Fig. 13**).

Isolate PE3 showed biocontrol activity against *M. phaseolina* (55%), *A. brassicae* (63%), *F. oxysporum* (60%). Isolate AF7 inhibited growth of *M. phaseolina* (48%) and *F. oxysporum* (51%) (**Fig. 13, Table 11**).

4.4.9 *Exopolysaccharide production*

Isolate PE3 was found to be an efficient EPS producer (comparing with other isolates), showing 0.54 ± 0.07 g/100 mL production under non-saline conditions. Highest EPS production of 1.25 ± 0.05 g/100 mL was observed at 2% NaCl which increased by 132% in comparison to non-saline control (**Table 9, Fig. 13**). Further increase in salinity reduced the EPS production to 0.55 ± 0.08 g/ 100 mL at 4% NaCl, 0.21 ± 0.03 g/ 100 mL at 6% NaCl and least production at 8% NaCl (0.06 ± 0.02 g/ 100 mL). Correlation coefficient (r) of -0.689 was obtained for EPS production versus salinity, further the model prediction through regression analysis was unable to establish relationship with salinity ($R^2 = 0.475$) (**Fig. 14 and 15**). The exponential increase in EPS production from non-saline to 2 % salinity and then again steep reduction in yield could possibly be the reason for failure of model prediction.

In comparison to PE3 (and other 42 isolates), AF7 was the most dominant EPS producer and there was an exponential increase in EPS synthesis when salt–stress was imposed. Maximum EPS production was reported at 2% NaCl, increasing significantly in comparison to non-saline condition (**Table 9, Fig. 13**), while the lowest production of 0.5 ± 0.12 g/100 ml was at 8% NaCl. The coefficient of determination (R^2) was equal to 0.151 and P value ~0.5 (**Fig. 14 and 15**) which suggests that no relation was established and further research is required to prove how salinity regulates EPS synthesis.

4.4.10 *Qualitative estimation of ACC deaminase activity*

For ACCD activity by isolates, the growth on DF medium with ACC as sole nitrogen source were checked. Both PE3 and AF7 showed growth on both plates with ACC and ammonium sulphate as nitrogen sources (**Fig. 13**).

4.5 Estimation of salt-tolerance traits of isolates

Salt-tolerance responses of selected isolate AF7 and PE3 was reported at various saline conditions and the change in behavior was statistically analyzed till 8% NaCl.

4.5.1 *Estimation of intracellular sodium ion accumulation*

In PE3 there was 133% increase in Na^+ accumulation when the cells were exposed to salinity (2% NaCl). The accumulation of sodium ions in cells were increasing along with salinity and was maximum at 6% NaCl (32591.72 µg/gm of cell dry weight) (**Table 10, Fig. 16**). Determining the relation, sodium accumulation was directly proportional to salinity with correlation coefficient (r) = 0.859 with a good-fit model (R^2 value 0.737) (**Fig. 14 and 15**).

Similarly, the amount of sodium accumulated in AF7 cells increased from 4292.21 µg/gm of cell dry weight in non-saline control to maximum of 34,024.57 µg/gm of cell dry weight at 6% NaCl. However, at 8% NaCl the sodium content decreased (26,465.53 µg/gm of cell dry weight (**Table 10, Fig. 16**). The sodium influx in AF7 cells was found to be directly related with salinity (correlation coefficient r =0.864, R^2 = 0.779) (**Fig. 14 and 15**) and therefore, the results are reflecting the direct conversation between bacteria and hyperosmotic surrounding.

4.5.2 *Endogenous accumulation of osmoprotectant proline*

In PE3 there was continuous increase in proline accumulation along with enhancing NaCl concentrations (upto 6% NaCl) (**Fig. 16, Table 10**). With the lowest accumulation under non-saline condition (103.71 ± 71 µg/ mg protein), the increasing trend of proline synthesis established a direct relationship with salinity corresponding to correlation coefficient (r) = 0.895 and the model supporting the hypothesis (coefficient of determination R^2 = 0.801) (**Fig. 14 and 15**).

AF7 showed proline accumulation at all salt levels (up to 8% NaCl) and there was 76% increase from non-saline conditions to 2% NaCl salinity and approximately 139% increase up to 8% NaCl (as compared to non-saline control). The coefficient of regression (R^2) = 0.89 and correlation coefficient (r) = 0.94 showed that there is a positive relationship between salinity and proline accumulation with a good-fit model.

4.5.3 ***Reduction potential of isolates***

The reducing power of PE3 in terms of quercetin equivalent (standard) was found to be maximum at 2% NaCl (3.45 ± 0.13 mg/ ml equivalent quercetin) increasing by 4.5% in comparison to non-salinized control (**Table 10, Fig. 16**). Above 4% NaCl, the property got reduced and minimum was found to be at 8% NaCl (1.95 ± 0.01 mg/ ml equivalent quercetin). A negative correlation coefficient (r) of -0.947 was reported with coefficient of determination (R^2) equivalent to 0.896 (**Fig. 14 and 15**).

Based on absorbance and comparison with standard (quercetin), reducing power of AF7 was found maximum at 2% NaCl and maintained significantly up to 6% NaCl and was least at 8% NaCl salinity (2.57 ± 0.19 mg/ml equivalent quercitin) (**Table 10, Fig. 16**). The coefficient of regression (R^2) was equivalent to 0.61, correlation coefficient (r) almost equal to 1 upto salt level 2%. However, an inverse correlation coefficient (r) = − 0.978 was obtained for higher salinity levels (4-8% NaCl) (**Fig. 14 and 15**).

4.5.4 ***Antioxidant activity as per DPPH assay***

The antioxidant activity of PE3 was maximum at 8% NaCl (87.96% inhibition of DPPH) and there was found to be a constant increase in the activity under salinity (**Table 10, Fig. 16**). Relatively, there was positive correlation between salinity and antioxidant activity of PE3 (correlation coefficient r = 0.983) and the perfect-fit model supporting the fact (R^2 = 0.966) (**Fig. 14 and 15**).

Explaining the DPPH assay, AF7 was able to inhibit DPPH radical by more than 65% and the property was found to be increasing with salinity. Maximum inhibition of 111% was reported at 8% NaCl by AF7. Therefore, there was found to be significant increase in antioxidant property of AF7 with the increase in salinity (*P= 0.01*) and a positive correlation (r = 0.96), the model of relativity was also proving the hypothesis (with coefficient of regression (R^2) equivalent to 0.92) (**Fig. 14 and 15**).

4.5.5 ***Hydrogen peroxide scavenging activity***

PE3 was a potent hydroxyl ion scavenger and the property was increasing along with increase in salinity. The maximum hydroxyl scavenging activity was at 8% NaCl (97.90%) and minimum at non-saline condition (83.03%) (**Table 10, Fig. 16**). There was positive relation reported between the scavenging ability and salinity with correlation coefficient (r) = 0.948 and corresponding R^2 value of 0.899 proving the model (**Fig. 14 and 15**).

Hydroxyl scavenging potential of AF7 was found to be increasing with salinity. Even under non-saline condition AF7 scavenged superoxide radical (generated from H_2O_2) by 81.07% and maximum of 96% at 8% NaCl. With coefficient of regression (R^2) as 0.91 and correlation coefficient (r) equal to 0.956 (**shown in Fig. 14 and 15**), the positive relation between salinity and ion scavenging activity of AF7 was established.

4.5.6 ***Biofilming ability***

Both PE3 and AF7 formed biofilm at all saline conditions. For PE3 the biofilming activity was found to be increasing with salinity and was maximum at 4% NaCl (OD 3.16 ± 0.033). There was about 14% increase in biofilm formation when salinity was increased from non-saline control to 4% NaCl condition (**Table 10, Fig. 16**). Interpreting from statistical analysis, biofilming ability of PE3 was directly proportional to salinity upto 4% NaCl; however, the property was indirectly

proportional to salinity upto 8% NaCl with correlation coefficient (r) of -0.801, however the model was poor fit (R^2 value 0.642) (**Fig. 14 and 15**).

The biofilming ability of AF7 was lesser than that of PE3, however with increasing salinity the value was also increasing and at 4% NaCl the value was maximum (OD 3.16 ± 0.089) and also equivalent to that of PE3. Statistical analysis revealed poor-fit model for salinity and biofilm formation of AF7 (R^2 value 0.035 and r = -0.188) (**Fig. 14** and **15, Table 10**).

4.5.7 *Biofilm formation on glass surface and visualization by Inverted Laser Scanning Confocal Microscopy (ILSCM)*

The selected isolate PE3 (on the basis of PGP properties and confining to the objectives of the study) was checked for biofilming on glass surface according to various salt-levels. Acridine orange is used as fluorescence dye to indicate biofilm biomass correctly as it is capable of binding to bacterial cells (both living and dead) as well as nucleic acid which is an important component of extracellular matrix (Branda et al. 2005). According to Strugger (1940), at pH of 5.7–8.0, staining with Acridine orange gives fluorescent green color for living cells and red color for dead cells. The visualization of biofilm through confocal microscopy revealed significant changes in clustering and biofilming activity upon increase in salt concentrations. Under non-saline conditions, the viable and living cells showed lesser clustering packed in microcolonies as compared to biofilms formed at 2%, 4% and 6% NaCl (**Fig. 17**). With increase in salinity the clustering of viable cells became more compact and denser, arranged in macro-colonies. The layer of EPS joining the bacterial colonies were clearly visible specifically in 2%, 4% and 6% NaCl exposed conditions. At 6% NaCl the cells were found to be elongated showing some sort of aggregation inside the matrix. Least or

almost no biofilming activity was determined at 8% NaCl, where few scattered bacterial cells were only visible with no EPS matrix.

4.5.8 ***Development of biofilm on root surface and analysis through SEM and ILSCM***

SEM analysis of biofilm formation on root surface revealed the presence of rod shaped PE3 colonies held together in a mucilaginous environment most probably of EPS and the associated matrix. Dense clusters of micro-colonies of PE3 were clearly detected when roots were treated with only bacteria (**Fig. 18**). Gummy layer binding the cells were also seen showing the production of EPS by PE3 for colonization. Roots treated with only EPS showed crystals of sugars sticking to the surface and further increasing the magnification it was found that the metabolite formed a thick layer on the roots due to formation of rhizoshealths. EPS plus bacteria also showed biofilm formation with bacteria encapsulated inside the matrix. Analyzing the roots at various magnifications, sugar crystals were seen sticking on the root surface. Numerous clusters of rod shaped bacteria (PE3) were observed and were covered with EPS (visible as sugar crystals) and extracellular matrix. Observing from the images it can be reported that the combination treatment had denser bacterial colonies and biofilm formation as compared to sole bacterial treatment. The negative control roots showed no presence of sugar crystals or bacteria confirming the authenticity of results and sterilization of roots.

Biofilming activity on root surface was also imaged through confocal microscopy. Viewing the images it was found that biofilm was formed in both the treatments *i.e.,* alone PE3 and the combination of PE3 and 2% EPS. Rod shaped green fluorescent bacteria in an encapsulated microenvironment elucidated the colonization of bacteria and the associated EPS matrix on the root surfaces. However, comparing the CFU (per gram of root) through *BioFilm Analyzer,* it was found that for only PE3 treatment the cell number was ~ 7.7×10^4 while for PE3 and EPS treatment the cells were 1.7×10^5

(per gram root). In both the treatments there were seen a protecting layer outside the bacterial colonies and even the EPS matrix was stained with green color against the black root background (**Fig. 19**).

4.6 Identification of bacteria

4.6.1 *Proteomic analysis through MALDI-TOF MS Biotyping*

Proteomic analysis of four selected isolates was done and diversity of bacteria was reported along with pseudomonads (**Table 12**). Isolate PE3 was found to be a match with *Pseudomonas putida* B401 UFL but with a score of 1.946. Therefore, through MALDI-TOF MS biotyping analysis PE3 was confirmed only at genus level *i.e.* belonging to *Pseudomonas.* **Fig. 20** represents the MALDI-TOF MS spectra of isolate PE3 showing protein profile (2-20 KDa).

MALDI-TOF MS biotyping of AF7 revealed that the bacteria best matched with *Alcaligenes faecalis* spp *faecalis* DSM 30030T (**Table 12**) with a score of 2.254 therefore confirming both the genus and species of the isolate. The protein profile of the bacteria is represented in **Fig. 21** showing the MALDI-TOF MS spectra.

MALDI-TOF MS biotyping results for identification of isolate CS5 and SS2 revealed that comparing with the Bruker taxonomy database both the strains were best matched with *Bacillus pumilus* DSM 27T DSM with a score of 1.97 for CS5 and 1.876 for SS2 (**Table 12**). Therefore, both these strains can be confirmed only at genus level (score < 2) *i.e.* belonging to *Bacillus.* **Fig. 22 and 23** represent the MALDI-TOF MS spectra of CS5 and SS2 respectively.

4.6.2 *Molecular analysis through 16S rRNA sequencing and phylogenetic tree analysis*

From the selected isolates four were analyzed through 16S rRNA sequencing and nucleotide homology (**Table 12**). The sequences were submitted to NCBI-GenBank

and respective accession number were assigned (**Table 12**). The 16S rRNA (~1200 bp) sequencing showed 100 % similarity of PE3 with *Pseudomonas entomophila* L48 (T) (accession number CT573326) through BLASTn and EzTaxon analysis and accession number for PE3 was MN515387 (**Fig. 24, Table 12**). Isolate PE3 was also submitted as *P. entomophila* at NAIMCC, India with accession number NAIMCC-B02324.

16S rRNA sequence of AF7 and AF2 showed 99% similarity with *Alcaligenes faecalis* strain NBRC 13111 (Accession Number NR_113606.1) both through NCBI-BLASTn and Ez-Taxon (**Fig. 25 and 26, Table 12**). The gene sequence (1340 bp) of AF7 is submitted to NCBI GenBank with accession number MK898928. *Alcaligenes faecalis* AF7 was subsequently submitted to NAIMCC, India with accession number NAIMCC-B-02325.

Molecular analysis of S1C through 16S rRNA sequencing showed nearest homolog with *Cellulomonas pakistanensis* NCCP-11 with only 92.78% identification score both through BLASTn and EzTaxon (**Table 12, Fig. 27**).

4.7 Characterization of exopolysaccharides under different saline conditions

Confining to the objectives of the study, EPS of *Pseudomonas* (isolate PE3) was selected for further characterization. EPS was extracted at various salt-concentrations (2-8% NaCl) and from non-saline control. After purifying the biopolymer, each EPS was checked for various properties.

4.7.1 *Compositional analysis*

(a) Colorimetric analysis

Estimating the carbohydrate content of PE3-EPS at various saline conditions, it was found that EPS extracted at 2% NaCl had maximum sugar content (9433.33 ± 154.56 mg/ gm of EPS) accounting for 51% increase as compared to non-saline control EPS (**Table 13, Fig. 28**). EPS obtained at 4% NaCl showed 6% increase in sugar content

comparing with non-saline control and least sugar content was reported in EPS produced at 8% NaCl.

Protein estimation of EPS elaborated similar trend of increase as that of carbohydrate content. Maximum protein content was at 2% NaCl (4469.67 ± 15.79 mg/ gm of EPS) which increased by 50% as compared to EPS under non-saline conditions (**Table 13, Fig. 28**). At 6% NaCl, EPS' protein content increased by 20% in comparison to EPS produced under non-saline conditions. Overall the protein content of EPS was far lesser than the carbohydrate content at all saline-conditions signifying the saccharide nature of the polymer.

The phenolic content of EPS was checked in order to determine the stress tolerance ability with increasing salinity. Phenolic content of EPS was maximum when the biomolecule was extracted at 2% NaCl (20.03 ± 1.53 mg/ gm) which increased by 27% as compared to non-salinized EPS (**Table 13, Fig. 28**). At 4% NaCl there was 5% decrease in phenolic content comparing with non-salinized control. Least phenolics were reported in EPS at 8% NaCl, decreasing by 16% in respect to non-salinized control and by 34% comparing with EPS at 2% NaCl.

Reducing sugar was reported only in non-salinized EPS and that extracted at 2% NaCl. Estimating from the color change (blue to green) the amount of reducing sugar was very less *i.e.* in the range 0.1-0.5 g.

(b) Detection of functional groups and monomeric unit of EPS

(i) Fourier Transformed-Infrared (FT-IR) spectroscopy

FT-IR analysis of extracted EPS was done and the effect of salt was checked on functional groups (**Fig. 29**). The broad stretching peak range 3600-3200 cm^{-1} in all EPS corresponded to hydroxyl group, characteristic of carbohydrate ring (Lim et al. 2005; Wang et al. 2015). The small peaks at 3000-2850 cm^{-1} in all EPS (except 8% NaCl-

EPS) denote CH stretching mainly of methyl or methylene groups often present in hexoses like galactose, glucose or deoxyhexoses (rhamnose, fructose) (Ismail and Nampoothiri 2010). Small peaks between 2500-2000 cm^{-1} in all EPS denoted the presence of free carboxyl groups (Osman et al. 2012), an important site for binding of cations (Wei et al. 2011). The peak range 1680-1640 cm^{-1} present in all EPS are the ring stretches of mannose or galactose or stretching vibrations of carbonyl groups in CONH moieties (Wang and Wu 2013; Zhang et al. 2017). The peaks in the region 1400 cm^{-1} to 1600 cm^{-1} (in all EPS) denoted amino and peptide content (Gutierrez et al. 2008; Zhang et al. 2019c). The peak at 1135.7 cm^{-1} was found only in non-salinized EPS and absent in all salt-exposed EPS (demarcated by arrow in **Fig. 29**). The peak corresponds to O-acetyl ester linkage bond of uronic acids (Bramhachari and Dubey 2006). The peaks around 1088 cm^{-1} to 1022 cm^{-1} were found in all EPS and mainly corresponds to P=O stretching (vPO2-) or polyphosphate proteins, nucleic acid phosphodiester and phosphorylated proteins (Yanikan et al. 2020). Further, peaks between 920-995cm^{-1} were present only in non-salinized and EPS extracted at 4% NaCl, indicating presence of gylcosidic linkage C–OC– (Yu et al. 2016). Band around 848.7 cm^{-1} characteristic of α-D glucan (Ye et al. 2009) was only present in non-salinized EPS. Peaks below 800 cm^{-1} indicate presence of alkyl halides (Nouha et al. 2016) present in all EPS.

(ii) Gas-chromatography mass spectrometry (GC-MS)

Detecting the monomeric unit through GC-MS, it was found that EPS extracted at all saline conditions consisted of melezitose as repeating unit (Retention time (RT) 7.813, 7.849, 7.992, 7.993, 8.001, 8.899, 8.946 min) (**Fig. 30**) which is being reported for the first time in any microbial EPS. Melezitose is an unusual trisaccharide and is composed of two molecules of glucose and one molecule of fructose. The structure of the

trisaccharide is D-glucopyranosyl (1→3)-β-D-fructofuranosyl (2→1)-α-D-glucopyranoside (Hehre 1953; Côté 2007). At different NaCl concentrations, the percent of melezitose (calculated from peak area) were 32.735% (non-saline EPS), 89.447 (2% NaCl-EPS), 64.983 (4% NaCl-EPS), 51.154% (6% NaCl) and 38.361% (8% NaCl-EPS). Additionally, all the EPS (except 8% NaCl-EPS) showed the presence of guanosine (RT around 8.1 min), which comprises guanine ring attached to ribose (ribofuranose) through β-N_9 glycosidic bond. Another change observed in composition of EPS upon exposure to salinity was the presence of additional sugars β-D-glucose and β-D-galactose (RT 8.906 min) only in 2% salinized EPS. 3-deoxy-D-mannoic acid (RT 8.842, 8.899 min) was found only in 4% and 6% NaCl-EPS. No detection of uronic acid may be due to failure of useful derivatives (Pitthard and Finch 2001). The non-carbohydrate unit of EPS consisted of proteins, esters (fatty acids) and carboxylic acid. Peak around RT 10.624 min detected in all EPS and another peak at RT 3.628 min in non-salinized EPS were unidentified and needs further characterization. Further it was noticed that fatty acids (esters) were increasing at higher NaCl concentrations (6-8% NaCl), which may be to maintain membrane stability under extreme conditions like salinity.

(c) Determining the structure and crystallinity through XRD

XRD patterns of EPS showed characteristic peaks at 30-35^o, 45-46^o, ~57^o, 65-67^o, 75-77^o 2 theta under all saline conditions (**Fig. 31**). The EPS extracted under non-saline conditions (with comparatively sharper peaks) had inter-planar spacing (d-spacing) between 1.26 and 2.8 [Å]. The d-spacing for EPS at 2% NaCl ranged 1.61- 2.8 [Å] and that for 4% NaCl-EPS, 6% NaCl-EPS and 8% NaCl-EPS ranged 1.96- 3.2 [Å], 1.4- 2.8 [Å] and 1.95- 2.8 [Å] respectively (specifically for sharper peaks). Further, calculating the crystallite size of each EPS, it was found that upto 4% NaCl the average size was

increasing; estimating to 18.306 nm at non-saline condition, 26.468 nm at 2% NaCl and 35.554 nm at 4% NaCl (**Table 14**), using Scherrer equation:

$$\mathbf{d} = \frac{K\lambda}{\beta cos\theta}$$

, where d is diameter of crystallite in nm

K is dimensionless shape factor, approximately equal to 0.9

λ is X-ray wavelength in radians

β is line broadening at half the maximum intensity (FWHM) in radians

θ is Bragg angle in radians.

However, at 8% NaCl, the size of EPS crystallite was decreased to 16.316 nm. Calculating the overall nature it was found that EPS was semi-amorphous and the degree of crystallinity was increasing with salinity upto 4% NaCl and then reduced at 6% and 8% NaCl. **Table 14** shows the amorphous and crystalline phases of EPS at various saline conditions. Another concept from the study highlighted that when comparing the diffractogram of NaCl crystals (JCPDS No. 5-0628) with EPS (at various saline conditions) it was found that the diffractogram patterns of EPS at 2% and 4% showed presence of NaCl (**Fig. 31**). But under non-saline condition and at 6% and 8% NaCl, the patterns were unmatched and this was almost in lineation with results of sodium accumulation by EPS (flame photometry).

(d) Structure morphology and elemental composition through SEM and EDS

The surface morphology EPS observed under SEM analysis revealed significant differences under various saline conditions. EPS extracted under non-saline conditions was rough with irregular lumps and crystals of sugars were visible. The matrix appeared compact and thick. Comparatively, the EPS matrix at 2% NaCl was thicker and denser and compactly bound sugar crystals were clearly visible in a lumpy manner. The

morphology of EPS at 2% NaCl was different from other EPS', and the observed viscosity of the matrix reported in SEM study elaborate it's role as thickening, stabilizing and biofilming agent along with water chelation potential. The EPS extracted under 4% NaCl showed irregular sugar matrix along with crystalline structures most probably of NaCl imbibed or trapped by the matrix. Strong binding between two phases (EPS and salt) was clearly visible. Similar type of trapping was observed in EPS extracted at 6% and 8% NaCl conditions. However, with increase in salinity the morphology changed from irregular matrix to more solid crystallized structures and surface covered with sugars. The density of EPS-matrix was found to be thinning above 4% NaCl (**Fig. 32).**

SEM-EDS was carried out to study the elementary composition of EPS under various saline conditions. The overall elements reported in all EPS were carbon (C), oxygen (O), sodium (Na) and chlorine (Cl). **Table 15** mentions the weight % and atomic % of EPS extracted at different salt-levels. The weight % of Na and Cl in EPS was found to be increasing along with salinity and was maximum at 4% NaCl. Evidently, under non-saline conditions 6.86 weight % of Na and 1.84% of Cl was reported, which increased to 19.11 weight % and 16.74 % of Cl in EPS extracted at 4% NaCl. With further increase in salinity the accumulation of NaCl was decreasing and minimum at 8% NaCl-EPS (**Fig. 33; Table 15**).

4.7.2 *Detection of salt-tolerance traits of EPS*

(i) Reduction potential

Determining the reduction potential, it was found that 0.02 mg/ ml of EPS (obtained from PE3 under non-saline conditions) exhibited the reducing power of 0.275 and EPS extracted at 2% NaCl had highest reduction potential of 0.805 (on the basis of absorbance). Calculating the quercitin equivalents from standard graph, the reducing

power of non-salinized EPS was found to be 850 mg/ gm which increased to 2241.667 mg/ gm at 2% NaCl stress. There was continuous increase in reduction potential of EPS (as compared to non-salinized EPS upto 6% NaCl) accounting to 164% at 2% NaCl, 106% for 4% NaCl and 19% increase at 6% NaCl (**Table 13, Fig. 28**).

(ii) Antioxidant activity

The antioxidant potential of EPS was also found to be increasing with salinity (upto 6% NaCl). EPS obtained under 2% NaCl conditions caused 83.72 % inhibition of DPPH (stable free radical) which was ~26% more than that by non-salinized EPS. Comparing with non-salinized EPS, 4% NaCl-EPS caused 8% more inhibition and 6% NaCl-EPS inhibited DPPH by more than 5% (**Table 13, Fig. 28**). Least inhibition was reported by EPS extracted at 8% NaCl (52.71 ± 2.76 %).

(iii) Hydroxyl scavenging activity

EPS obtained at 2% NaCl was found to be very efficient ROS scavenger with the highest hydroxyl scavenging activity of 87.75 %. The hydroxyl scavenging activity of EPS increased till 4% NaCl (in comparison to non-salinized EPS). The maximum increase was by 9% for 2% NaCl-EPS, followed by 2% increase in 4% NaCl-EPS as compared to EPS at non-saline conditions (**Table 13, Fig. 28**). Minimum scavenging activity was reported in 8% NaCl-EPS which reduced by about 12% with respect to non-salinized EPS.

(iv) Flocculation activity

The formation of soil aggregates by EPS was measured as the function of flocculation activity. Flocculation activity by EPS obtained from PE3 under non-saline conditions was found to be 68.44 %, which increased by about 3% in 2% NaCl-EPS. As compared

to non-salinized EPS, 4% NaCl-EPS had 1% more flocculation, but the activity was reduced in further salinized EPS (i.e. at 6% and 8% NaCl) (**Table 13, Fig. 28**).

(v) Emulsification activity

The formation of emulsions with organic solvents such as olive oil was determined in terms of emulsification index. EPS obtained at 2% salinity stress was found to be a potent emulsifier and index of 57.75 % was found in comparison to 53.32 % by EPS obtained in non-saline conditions (increase% = 8%). Although EPS extracted at 8% NaCl was also an emulsifier but the potential was least as compared to other EPS' (**Table 12, Fig. 31**).

(vi) Accumulation of sodium ions

EPS was also found capable of chelating sodium ions under saline conditions and the highest amount of chelation reported after 24 h was 37,525 mg Na^+ per kg (dry weight of EPS) by 2% NaCl-EPS. Non-salinized EPS chelated very less quantity of sodium (1863 mg kg of dry weight) which could most probably be due to adsorption of salts from culture-medium. In comparison to non-salinized EPS, the chelation activity was 19, 16, 14, and 11 fold higher in EPS extracted at 2%, 4%, 6% and 8% NaCl respectively (**Table. 12, Fig. 31**).

4.8 Effect of EPS and selected isolates on sunflower growth, yield and salt-tolerance traits

4.8.1 *Preparation of bioformulation*

Talc-based bioformulation of PE3 and AF7 was prepared and amended with EPS at various concentrations (0.1, 1, 2, 5% w/v) under aseptic conditions. The respective treatments were checked by pot and field trials for two years and the difference in

growth and salinity responses of plants were determined in comparison to untreated plants.

(a) *In vitro* study

In pot-trial there was significant difference in germination rate of plants. There was linear increase in germination rate with increase in EPS concentration. Considering effect of only EPS, treatments T3 and T4 showed maximum germination rate of 67%. However, addition of bacteria along with EPS showed better results with 73% germination rate for both AF7 and PE3 (T5 and T6). Least effective result was obtained in plants treated with 0.1% EPS (**Table 16**).

Root colonization by PE3 and AF7 was checked in specific treatments (T5-T8) at various growth stages and comparative analysis was done to show the effect of EPS in colonizing bacteria. Isolate PE3 was found to be a potent colonizer, relatively 30 DAS the population density was found to be 6.519 log10 CFU/ gram of root, which increased to 7.623 at 60 DAS and further the values recorded were 6.707 and 4.398 log10 CFU at 90 and 120 DAS (treatment T8) respectively. In treatment T6 with addition of EPS, the colonization of PE3 increased with value 8.623, 8.898, 7.827 and 5.748 log10 CFU per gram of root at 30, 60, 90 and 120 DAS respectively.

Estimating the colonization potential of AF7 in treatments T6 and T7 it was found that the bacteria successfully colonized the roots. Log10 CFU value/ gram of root of 5.447, 6.653, 5.85 and 5.255 at 30, 60, 90 and 120 DAS respectively was found for T7 treatment (AF7 only). However, with the addition of EPS to AF7 bioformulation (T6), the colonization was enhanced and the maximum population density of 8.362 log10 CFU value/ gram of root was at 60 DAS. The population density at 30, 90 and 120 DAS was 7.623, 8.079 and 6.491 log10 CFU value/ gram of root respectively. **Fig. 34** shows the phylogenetic tree of PE3 and AF7 obtained from the treated plants.

4.8.2 (a*) Analysis of growth parameters of sunflower plants*

The growth parameters of treated plants showed significant difference as compared to untreated control. It was found that EPS was potent in promoting plant growth, and higher concentration of EPS (2% and 5% EPS) caused significant increase in plant growth than bacteria alone (in comparison to control plants). The treatment with 0.1% EPS was least effective and 2% and 5% EPS treatments showed almost similar results and therefore, further EPS concentration was not checked. Additionally, with the supplementation of EPS, there was highest increase in all growth parameters reported (**Table 16, Fig. 35**). Further, PE3 was a better plant growth promoter as compared to AF7. Comparing the difference in root length with subjection to various treatments highest increase of 179% was reported for 2% EPS plus PE3 (T6) treated plants which was 4% more than EPS and AF7 combination treatment (T7). Shoot length also increased by about 2-folds when plants were treated with EPS and PE3/ AF7 combination. The combination treatment (EPS + PE3/ AF7) was 50% more efficient than only EPS treatments (for 2% and 5% EPS treatment) and 67% more than only bacterial treatment (**Table 16, Fig. 35**). Fresh weight of the plant was also correspondingly increasing with higher concentration of EPS and was almost similar when plants were treated with 2% and 5% EPS. The fresh weight increased by about 30% in presence of 2% and 5% EPS treatment as compared to untreated control. The highest increase was reported for EPS (extracted from PE3) and PE3 treatment with 35% increase comparing with untreated plants and the treatment was 2% more efficient than EPS plus AF7 inoculated plants (**Table 16**). Considering only bacterial treatment, PE3 inoculated plants showed 15% increase in fresh weight, while AF7 caused increment by 10% (in respect to untreated plants). Similarly, the dry weight of the plant was highest for 2% EPS and PE3 (combination) treated sunflower plants, corresponding

to 86% increase in comparison to untreated plants. Lowest effect on plant dry weight was by 0.1% EPS (T1) which was still 44% more than untreated plants. PE3 caused 3% increase in dry weight as compared to AF7. Explaining the effect of treatments on stem girth and head size, there was significant difference when EPS and bacteria were added to plants. The head size increased from 1.63 cm (in control plants) to 4.43 cm when 2% EPS and PE3 were added to plants. All the treated plants showed more than 50% increase in head size as compared to control plants. The stem girth also increased by more than 20% in treated plants in comparison to untreated plants. Considering the seed yield there was more than 49% difference between the yield of untreated plants and those supplemented with bacteria and EPS. The combination treatment of EPS and PE3 was most effective with the highest yield of 8.33 g/ pot. The combination treatment showed more than 34% increase in yield as compared to sole EPS treatment (5% EPS), and also a difference of more than 74% was reported between combination treatment and only bacterial treated plants (**Table 16**).

4.8.3 (a) ***Analysis of physicochemical and biochemical properties of sunflower plants***

The chlorophyll content increased in treated plants in comparison to untreated control. The highest increase of 62% was reported in 2% EPS and PE3 (combination) treated plants while minimum increase of 20% in 0.1% EPS treated plants as compared to untreated salt-stressed plants (**Table 17, Fig. 36**). The combination of AF7 and 2% EPS increased the chlorophyll content by 59% which was 39% more than only AF7 inoculated plants. PE3 was a better growth promoter than AF7 and relatively increased the chlorophyll content by 1% more than AF7 treated plants. Similar trend was observed in case of carotenoid content of plants. The highest increase in carotenoid content was by 75% in EPS plus bacterial treatments (comparison to untreated control). Treatment with high concentration of EPS caused 18% more increase in carotenoid

content as compared only bacterial treatments (**Table 17, Fig. 36**). Minimum increase in carotenoid content was by 0.1% EPS (T1) which was still 31% higher than untreated control plants. Flavonoid content in salt-stressed uninoculated plants was highly depressed and the property showed elevation with application of bacteria and EPS. With 49% and 48% increase in flavonoid content in PE3 + EPS (T5) and AF7 + EPS (T6) treated plants respectively, the two treatments were categorized as best growth stimulators. Treatment with only AF7 and 1% EPS caused almost similar effect, increasing the flavonoid content by 13% (**Table 17, Fig. 36**). Treatment by 2% and 5% EPS increased the flavonoid content by 21% as compared to untreated control. Further, measuring the phenolic content of plants, the combination (EPS and bacteria) showed best results increasing the phenolics in plants by 80% and 78% for PE3 plus EPS and AF7 plus EPS respectively (**Table 17, Fig. 36**). There was insignificant difference between 2% and 5% EPS treated plants showing phenolic elevation by 55% and 54% respectively. Protein content was also increasing upon treatment with bacteria and EPS. AF7 showed 37% increase while PE3 caused elevation by 40% in protein content as compared to control plants. However, 2% and 5% EPS treatments showed better results with 59% increase in protein content of leaves in comparison to untreated control plants. Additionally, the combination of EPS and PE3/ AF7 caused highest increase in protein content by more than 74% (**Table 17, Fig. 36**). The highest impact of treatments was on the sugar content of the plants. Treated plants showed significant changes in carbohydrate content with minimum increase of 156% as compared to uninoculated control. In EPS plus bacteria (PE3/ AF7) treated plants, the increase in carbohydrate content was more than 4-folds (best treatment). Both 2% and 5% EPS treated plants showed sugar content elevation by 2.8-folds as compared to control plants which

marked significant difference in the results and was most probably salinity-guided (**Table 17, Fig. 36**).

4.8.4 (a) ***Analysis of salt-tolerance traits of sunflower plants***

Maximum tolerance behaviour of plants was in EPS and bacterial combination treatments (T5 and T6). Accumulation of osmoprotectant in plants was highly induced with application of EPS and bacteria. There was more than 1.7-fold increase in proline content of plants when treated and the maximum proline content was reported in EPS and PE3/ AF7 applied plants, corresponding to 2.5-fold increase as compared to untreated plants (**Table 18, Fig. 36**). Similarly, there was also significant difference in reduction potential between treated sunflower plants and untreated stressed plants. The maximum reducing power increase was found in EPS and PE3 treated plant, which was 36% more than only EPS treatment (2% EPS) and 68% more efficient than only PE3 inoculated plants (**Table 18, Fig. 36**). The antioxidant activity and hydroxyl scavenging activity in plants was showing similar trend upon inoculation, with minimum effect by 0.1% EPS and maximum by EPS and bacteria combination. Highest inhibition of DPPH and super peroxide radical was reported in treatment T6 (PE3 + 2% EPS) accounting to 53.39% and 57.77% respectively. The effect of PE3 and AF7 (only) treatments on ROS scavenging was almost equivalent to 2% EPS treatment ($P < 0.05$) (**Table 18, Fig. 36**). Lowest inducer was 0.1% EPS treatment with DPPH activity 25.49% and hydroxyl scavenging activity 32.3% and the control plants showed the value of 22.88% for former and 20.86% for latter. RWC defines the stability of plants against salinity. Therefore, comparing with untreated plants EPS and PE3 maintained RWC as 96.65% and EPS (2%) along with AF7 maintained the turgidity to 94.10% (**Table 18, Fig. 36**). Higher concentration of EPS (2% and 5%) caused 50% increase in RWC as compared to control plants. PE3 and AF7 influenced plant water level with an increase of 41%

and 40% respectively, comparing to untreated control. Electrolyte leakage and linked membrane stability was highly correspondent to PGPB and metabolite inoculation. Lesser the electrolyte leakage more is the membrane stability and stress-resistance in plants. Least electrolyte leakage with maximum membrane stability index was reported in EPS and PE3 treated plants corresponding to 51% reduction in EL and 1.9-fold increase in MSI as compared to untreated control (**Table 18, Fig. 36**). Treatment T1 showed least effect with 26% reduction in EL and 76% increase in MSI in comparison to control plants. PE3 and AF7 showed similar influence on EL (33% reduction) and MSI (125% and 124% respectively) contrasting with control plants.

(b) ***In vivo study***

In field conditions, treated plants showed significant difference in plant growth as compared to untreated control. **Fig. 38** shows the barren condition of saline lands in Ambedkar Nagar, Uttar Pradesh (experimental field). Both AF7 and PE3 were found to be good colonizers of sunflower roots even in field conditions. However, addition of EPS helped in maintaining the bacterial population in the rhizosphere. For treatment T5 (2% EPS + PE3), the population density of 7.99, 8.81, 6.97 and 5.68 log 10 CFU was maintained at 30, 60, 90 and 120 DAS respectively. Comparatively, lesser CFU was reported for treatment T8 (PE3 only) with maximum colonization of 7.51 log 10 CFU at 60 DAS and minimum of 3.04 log 10 CFU at 120 DAS. For treatment T6 (AF7 + 2% EPS) the recorded population density of the isolate AF7 was 6.91, 8.59, 6.46, 5.73 log 10 CFU after 30, 60, 90 and 120 DAS respectively. The decrease in bacterial population at the time of harvesting was more prominent in only bacterial treated plants as compared to EPS and bacterial combination. The results clearly indicated the role of EPS in maintaining the population of inoculated bacteria under saline conditions for

longer period of time. **Fig. 34** shows the phylogenetic tree of PE3 and AF7 obtained from the treated plants under field conditions.

Salinity inhibited the germination of seeds and in uninoculated set, germination rate of only 30 % was reported while treatments safeguarded the seed viability and enhanced the germination rate. Highest germination rate of 81 % was in plants treated with the combination of 2% EPS and PE3/ AF7. Least effective treatment was 0.1% EPS (T1) with germination rate of 61% (**Table 19, Fig. 37**).

4.8.2 (b) *Analysis of growth parameters of sunflower plants*

It was observed from the results that the inoculation of plants with EPS and bacteria (in combination) significantly increased the growth parameters in comparison to uninoculated control. Highest increment in root and shoot length was reported for treatment with 2% EPS and PE3 corresponding to 49 % and 85 % respectively (in comparison to control). EPS and AF7 increased the root and shoot length by 47% and 85% respectively. The lowest increase in root length was found for 0.1% EPS treatment with 23% increase (**Table 19**). Plant biomass was negatively affected by salinity, and in correspondence the untreated plants showed minimum fresh and dry weight (84.43 g and 64.46 g respectively). The trend of biomass enhancement in treated plants was according to the increase in EPS concentration and maximum biomass was reported in treatment with metabolite (2%) and bacterial combination (PE3/ AF7) (fresh weight 322.12 g and dry weight 266.17 g) (**Table 19, Fig. 38).** Stem girth was also measured to estimate the health of plants in saline soil. Again the combination (EPS and bacteria) was the highest stimulator, increasing the stem girth to 7.17 cm, comparing with minimum data recorded in control (2.29 cm). AF7 and its EPS combination increased the stem girth by 182% in comparison to control plants (**Table 19).** The results also clearly elucidated that increasing concentration of EPS (i.e. 2% and 5% EPS)

correlatively enhanced the plant growth parameters (by 179% and 180% respectively). The combination treatment showed 51 % (for PE3) and 40% (for AF7) increase in stem girth over sole bacterial treatment. The productivity of sunflower is highly dependent on the size of head bearing the seeds. The results obtained in the study showed significant difference in head size between untreated plants and EPS and bacteria inoculated sets. Salinity impacted the head size with mere 3.65 cm head in case of control. Treatment of bacteria along with 2% EPS increased the head size by more than 2-fold in comparison to untreated control (**Table 19, Fig. 38).** The lowest concentration of EPS (0.1 %) showed minimum improvement in the head size (10.23 cm), while the highest EPS concentrations (2% and 5%) showed almost similar increase (12.11 cm and 12.17 cm respectively). The increasing head size by various treatments resulted in corresponding increase in the seeds per plant and oil content of the sunflower. **Table 19** shows the effect of various treatments on growth parameters of plants.

4.8.3 (b) ***Analysis of physicochemical and biochemical properties of sunflower plants***

The qualitative physicochemical analysis of leaves revealed the production of saponins, polyphenols and steroids in plants but no tannin production was reported (**Fig. 39**). The biochemical properties of sunflower plants also showed drastic effect when treated with metabolite and bacterial combination (**Table 20**). The chlorophyll content of sunflower leaves was enhanced when treated with 2% EPS, 5% EPS and bacteria in combination with 2% EPS (**Table 20, Fig. 39**). Highest increase of 205% and 206% in chlorophyll content was reported when 2% EPS was applied along with AF7 and PE3 respectively. Individual application of bacteria and metabolite although enhanced the chlorophyll content yet there was a difference of about 5% (for only EPS treatment) and 23 % (PE3) and 27% (AF7) (for sole bacterial application) compared with combination (2% EPS

plus bacteria). Similarly, carotenoid content of leaves was also enhanced in presence of different treatments of EPS and bacteria.

Significant reduction in carotenoid content of plants was found due to salt toxicity and analogously the untreated plants exhibited the lowest amount of 0.108 mg/gm. Considering the treated plants, the trend of increase in carotenoid content was, 0.1 % EPS < AF7 only < PE3 only < 1% EPS < 2%, 5% EPS (almost equivalent) < 2% EPS and AF7 < 2% EPS and PE3 combination (**Table 20, Fig. 39**). The leaves of salt stressed sunflower were checked for flavonoid content and there was increase in case of all the treated plants. Among the treatments, the higher concentration of EPS (2% and 5%) were more influential in increasing flavonoid content compared with 0.1 % and 1% EPS (**Table 20, Fig. 39**). Relatively, there was about 16% increase in flavonoid content of plants treated with 2% and 5% EPS as compared to 0.1 % EPS treatment. The maximum increase in flavonoid content (of 58 %) was found for metabolite and PE3 inoculated plants followed by 51% increase in EPS and AF7 applied plants in comparison to untreated control. Both the bacteria, AF7 and PE3, increased the flavonoid content of plants by 28% (compared with control). With 24% increase in flavonoid content as compared to untreated control, 0.1% EPS was least effective treatment.

The phenolic content in plants signify the induction of salt-tolerance responses and enhanced survivability. Higher concentration of metabolite was found to be more efficient in increasing the phenolic compounds as compared to only bacteria-treated plants (increased by 111-113%) (**Table 20, Fig. 39**). The phenols accumulated due to PE3 and AF7 inoculation (in plants) amounted to 192 µg/ mg and 189.5 µg/ mg respectively, while for treatments with higher EPS concentration (2% and 5%) the content was 280 µg/ mg and 283 µg/ mg respectively. The synergistic effect of PE3/

AF7 and EPS was the highest stimulator resulting in 328/ 309.2 µg/ mg phenolic content (**Table 20, Fig. 39**). Maximum carbohydrate content of 845 mg/g was found in the plants treated with combination (EPS and PE3) and it was about 7–8 % higher than only EPS treated plants (2% and 5% EPS) (**Table 20, Fig. 39**). AF7 and 2% EPS also increased the carbohydrate content by 4-fold against 2.8-fold increase by only AF7 treatment (T7) in comparison to untreated control (**Table 20, Fig. 39**). Inoculation of plants also triggered the protein content and again combination of EPS and PE3 showed highest impact (79% increase) (**Table 20, Fig. 39**). EPS concentration of 0.1 % caused 19 % increase, while 2% and 5% increased the protein level by about 69–71 %, the combination of EPS and AF7 showed increase of 74 % and only PE3 elevated the content by 27% as compared to uninoculated control.

4.8.4 (b) ***Analysis of salt-tolerance traits of sunflower plants***

Further, the salt tolerance traits and defensive responses in plants were found to be triggered due to EPS application. In case of the salt tolerance-properties (in plants); reducing power, hydroxyl scavenging activity, antioxidant activity and osmolyte accumulation were significantly stimulated in case of 2% and 5 % EPS and combination of EPS and bacteria (**Table 21, Fig. 39**). The most interesting results were found for osmolyte accumulation (proline). The untreated control had the least osmolyte content (251 µg/ gm) thereby pertaining to lower survivability rate of plants. EPS and bacteria in combination, triggered highest proline accumulation in plants in response to salt stress (682 and 673.52 µg/ gm). The least influencing treatment was 0.1 % EPS and PE3 (alone) accounting for 477 µg/ g and 483 µg/ g proline content respectively (**Table 21, Fig. 39**).

The reduction potential of plants increased by about 78/ 79 % in EPS and AF7/ PE3 (combination) treated plants as compared with untreated control (**Table 21, Fig. 39**).

The lowest increase of 49% was found in plants treated with 0.1% EPS contrasting to untreated control plants. PE3 led to better improvement in reduction potential of plants as compared to AF7 (difference of 4%). Similarly, hydroxyl scavenging and antioxidant activity of plants also enhanced more with higher EPS concentrations. In comparison to control plants, 0.1% EPS showed 37% inhibition of DPPH and 48% scavenging of superoxide radical (**Table 21, Fig. 39**). Further, comparing the effect of higher concentration of EPS (5%) with PE3 treatment, there was about 8% (for antioxidant activity) and 13 % (hydroxyl scavenging activity) difference. (**Table 21, Fig. 39**).

4.9 Statistical analysis of results and correlative analysis of various treatments

(a) *In vitro study*

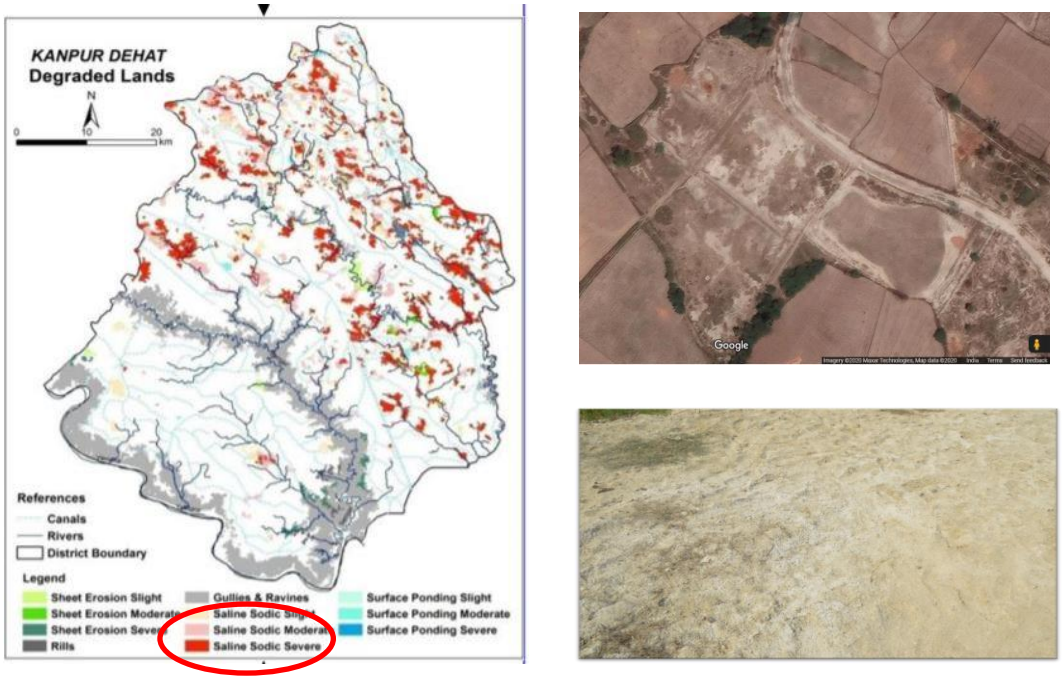
The effect of EPS and bacteria on various growth parameters and salt-tolerance properties of sunflower was analyzed using heat map and clustering. The dendrograms of heatmap for pot study (**Fig. 40a**) describes two major clusters: one showing lesser effect and the other with major impact on plant growth, biochemical and salt-tolerance properties. Elaborating the two clusters, the combination of EPS (2%) and bacteria (PE3/ AF7) and higher concentration of EPS (2% and 5%) were the major plant growth and salt-tolerance stimulators. In the same cluster, 2% and 5% EPS showed almost insignificant difference ($P< 0.05$), however, the combination was clustered as the outstanding treatment with highest impact on plants. In the other cluster (with lesser effect) there were reported two subgroups: firstly the treatment of bacteria alone (T7 and T8) and 1% EPS treatment (T2) and secondly the untreated control and treatment with least effect on plant growth *i.e.* 0.1% EPS (T1). Interestingly it was found that effect of bacteria (both PE3 and AF7) was almost similar to that caused by 1% EPS treatment (T2).

Correlogram computing Pearson correlation coefficient ($P < 0.05$) was drawn to determine the effect of increasing concentration along with properties of EPS on salt tolerance traits of inoculated plants (**Fig. 40b**). Almost all the salt tolerance traits (except EL) of plants were positively stimulated with increasing EPS concentration, thereby elucidating the role of EPS in increasing stress resilience in plants (correlation coefficient r range 0.51 to 0.71). Also the model predicted from the correlation graph signifies that the salt tolerance properties induced in plants due to EPS were inter-related (r range between 0.74 and 1.00) and this may be due to some common signals or molecular interactions between the pathways requiring further study.

(b) ***In vivo study***

Explaining the heatmap and clustering it was found from the dendrograms that there were two major-clusters including more potent treatments (2% and 5% EPS; EPS plus bacteria) and the other with comparatively lesser influence on plant growth and tolerance (0.1 and 1% EPS, bacteria only and control) (**Fig. 41a**). Among the lesser impactful treatments, the untreated control (T9) showed significant difference against the treatments. Treatment T3 and T4 were insignificantly different and the combination of bacteria and EPS were the most effective treatments (T5 and T6) and significantly more than all the other treatments.

The role of EPS in influencing salt-tolerance traits of sunflower was checked through correlogram (**Fig. 41b**). Similar, to the observations from pot study, EPS was found to play a significant role in improving salt-tolerance traits of sunflower plants. Almost all the salt-tolerance properties of plants were directly correlated to EPS concentration (correlation coefficient (r) 0.62 to 0.79). Comparing the salt-tolerance properties of plants, the correlation coefficient (r) ranged from 0.54 to 0.99, which showed that there was some sort of relationship found among them.



Degraded lands in Kanpur Dehat, Uttar Pradesh, India
(https://www.nbsslup.in/assets/uploads/clinks/Land%20Degradation%20Assessment%20Using%20MODIS%20NDVI%20Time%20Series%20Data.pdf)

Fig. 8 Details of sampling sites: Kanpur Dehat, Uttar Pradesh, India (20°38 E and 80°21 N)

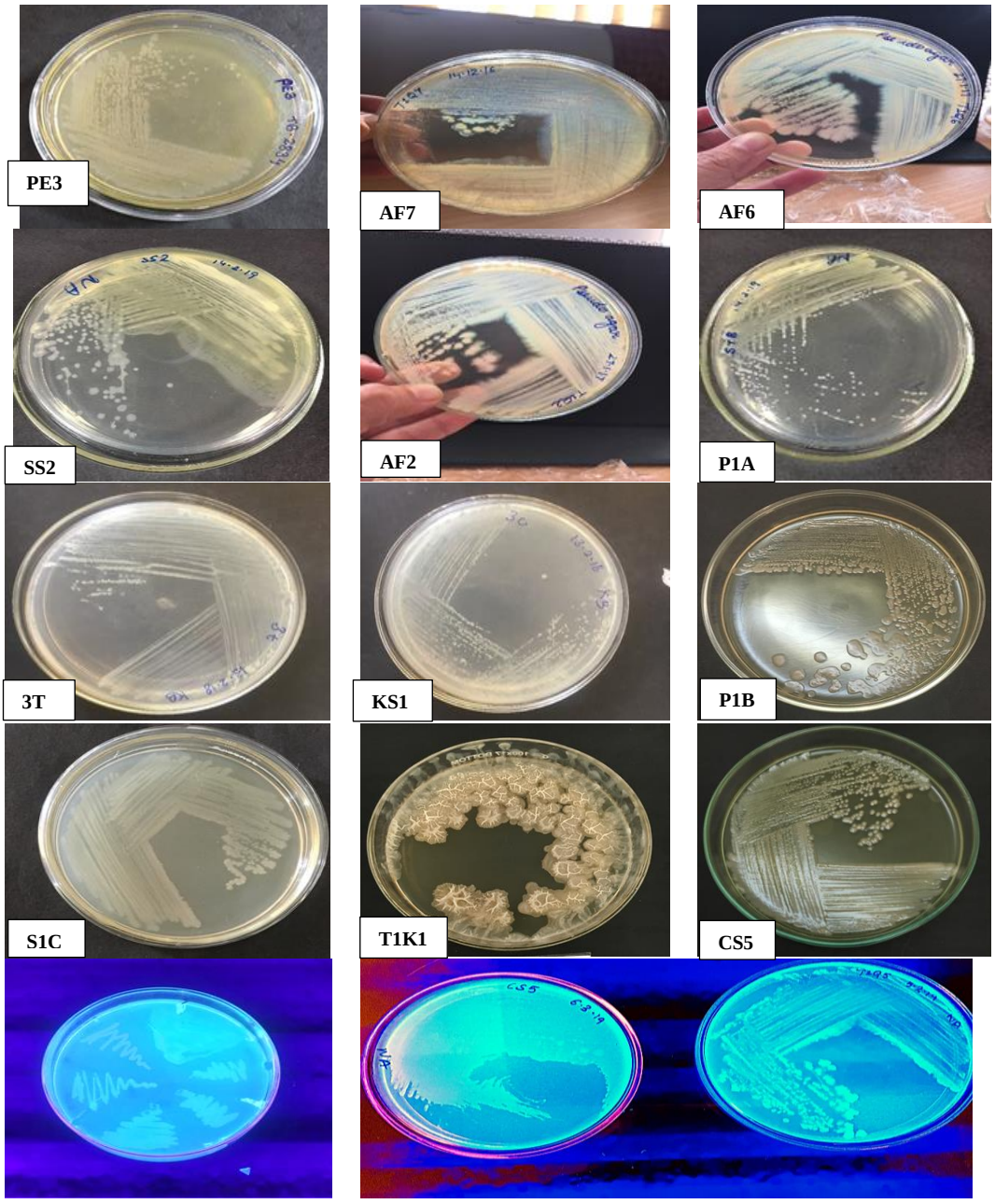
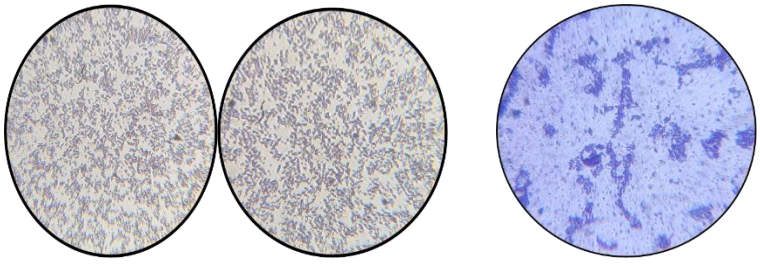
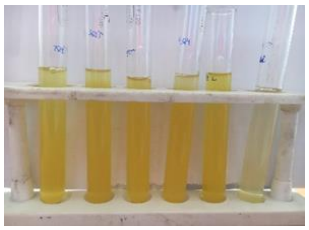


Fig. 9 Growth of selected isolates on Nutrient agar and Pseudomonas agar media (fluorescein) and fluorescence of the colonies under UV light

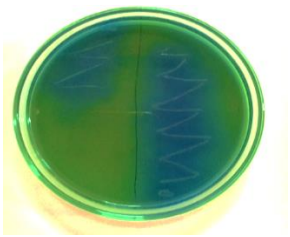


Gram positive **Gram negative**

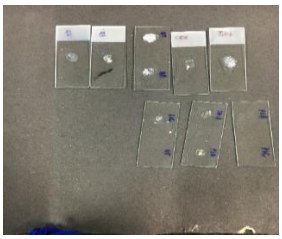
Fig. 10 Gram staining of bacterial isolates



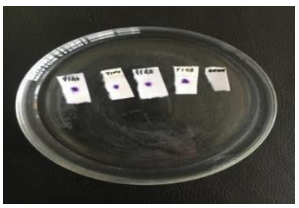
Ammonia production



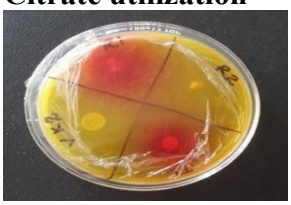
Citrate utilization



Catalase test

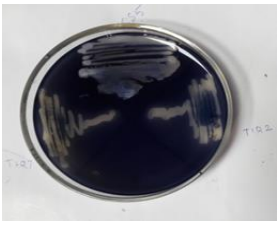


Oxidase test

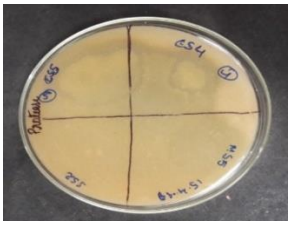


Urease test

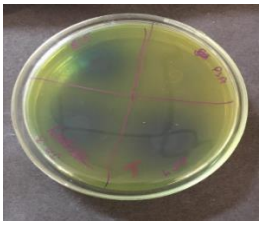
Cellulase test



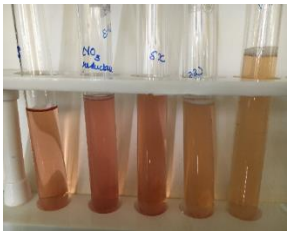
Amylase test



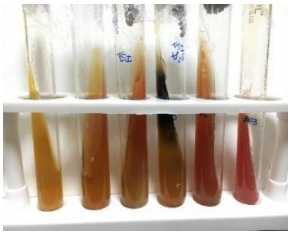
Protease test



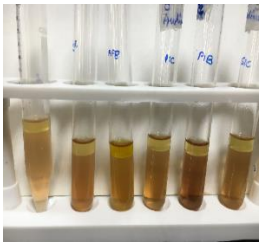
Lactose fermentation



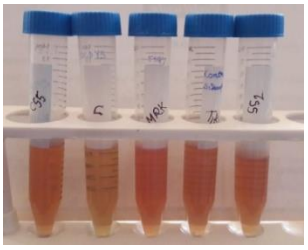
Nitrate reductase



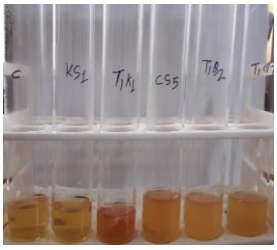
H_2S production



Indole production

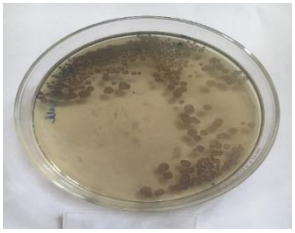


MR-VP test

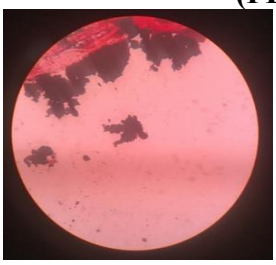


Gelatinase test

PHB accumulation (PE3)



PHB accumulation (AF7)



Sudan black stained colonies due to PHB granule accumulation

Fig. 11 Biochemical characterization of selected isolates

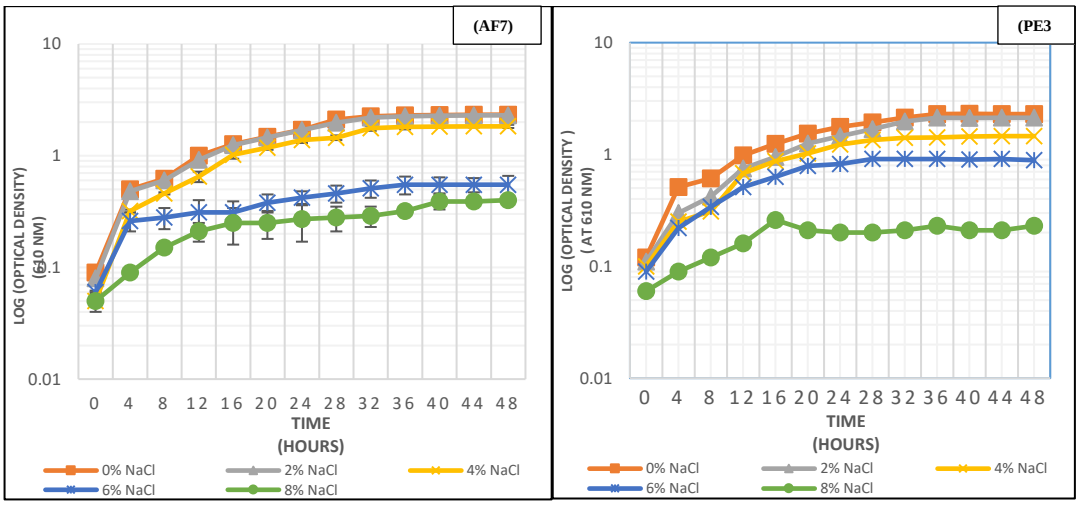
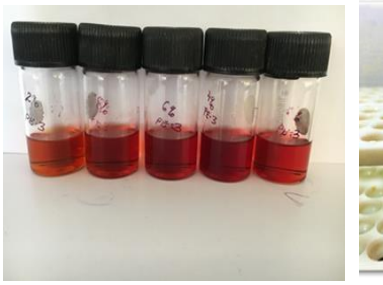
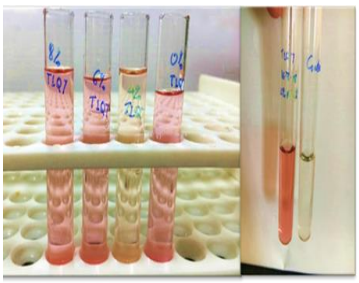


Fig. 12 Growth curve of isolates PE3 and AF7 under different salt concentrations (2-8% NaCl) and non-saline control

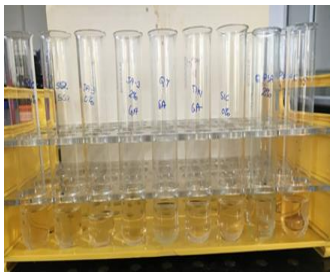
Data are represented as mean values (n = 3); error bars represent standard deviation



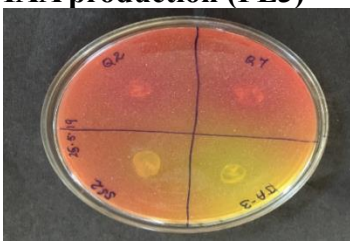
IAA production (PE3)



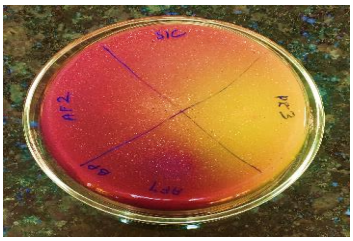
IAA production (AF7)



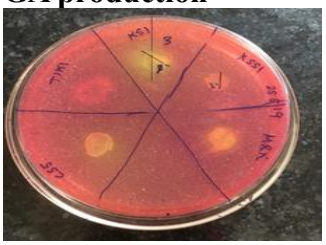
GA production



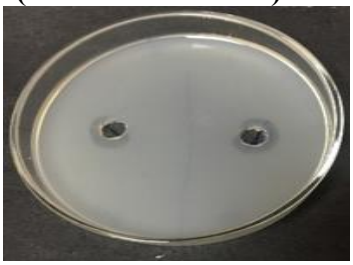
Potassium solubilization (non-saline control)



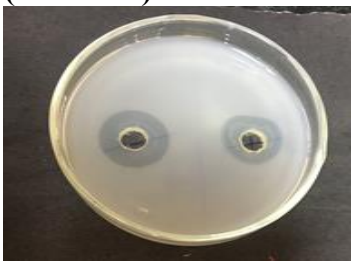
Potassium solubilization (2% NaCl)



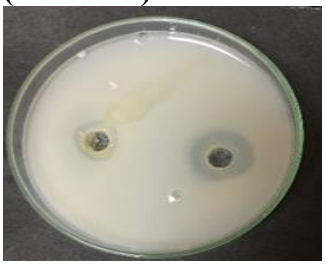
Potassium solubilization (4% NaCl)



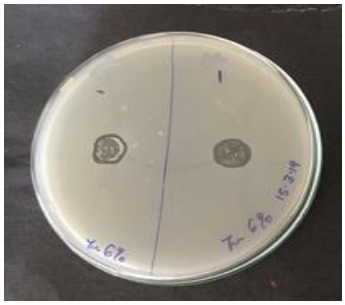
Zinc solubilization (non-saline control)



Zinc solubilization (2% NaCl)

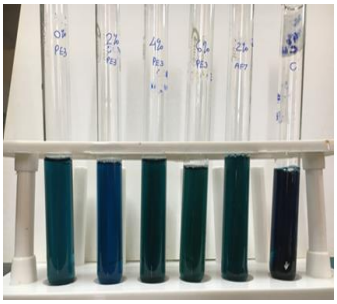


Zinc solubilization (4% NaCl)



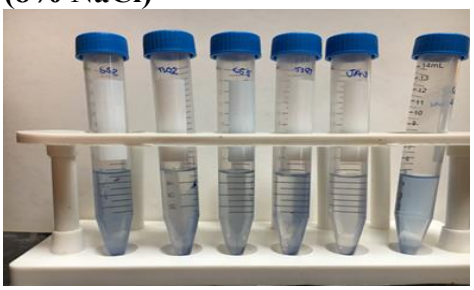
Zinc solubilization (6% NaCl)

Zinc solubilization (8% NaCl)

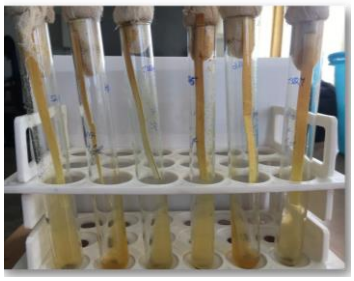


Phosphate solubilization

Siderophore production (Plate assay)

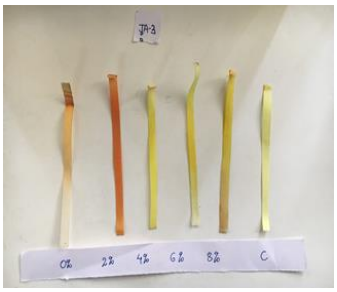


Siderophore production (CAS-Shuttle assay)



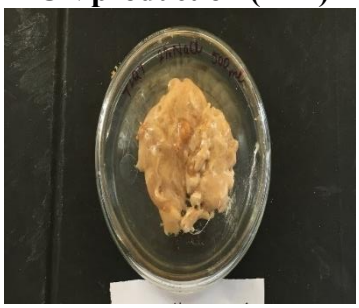
HCN production

HCN production (AF7)

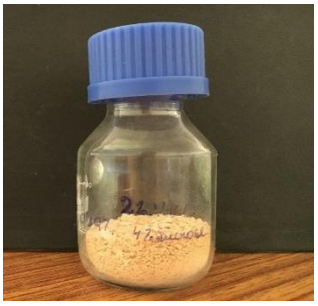


HCN production (PE3)

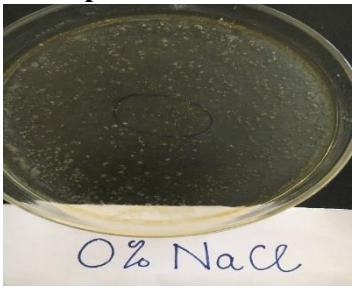
EPS production



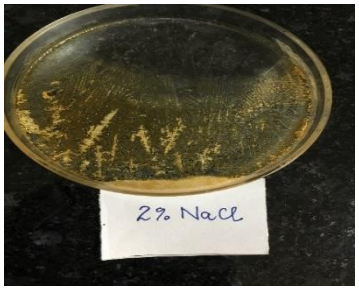
Extracted EPS



Purified and dried EPS

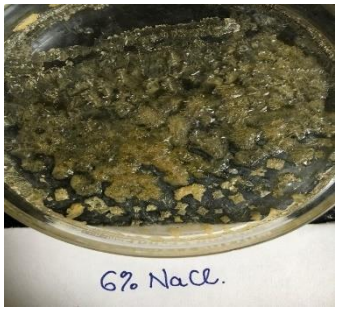


EPS under non-saline condition



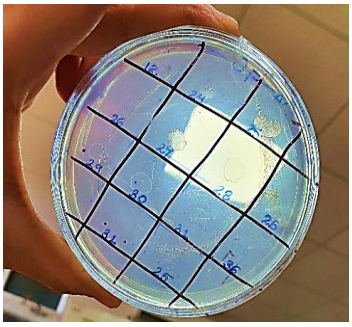
EPS at 2% NaCl

EPS at 4% NaCl

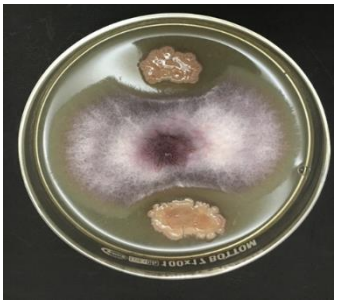


EPS at 6% NaCl

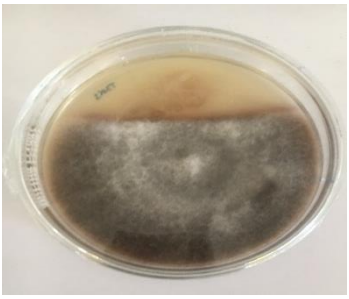
EPS at 8% NaCl



ACC deaminase activity



Biocontrol against *Fusarium solani*



Biocontrol against *Alternaria brassicae*

Biocontrol against *Macrophomin a phaseolina*

Biocontrol against *Fusarium oxysporum*

Fig. 13 Plant growth promoting properties of isolates under various saline conditions and their biocontrol activity against common phytopathogens

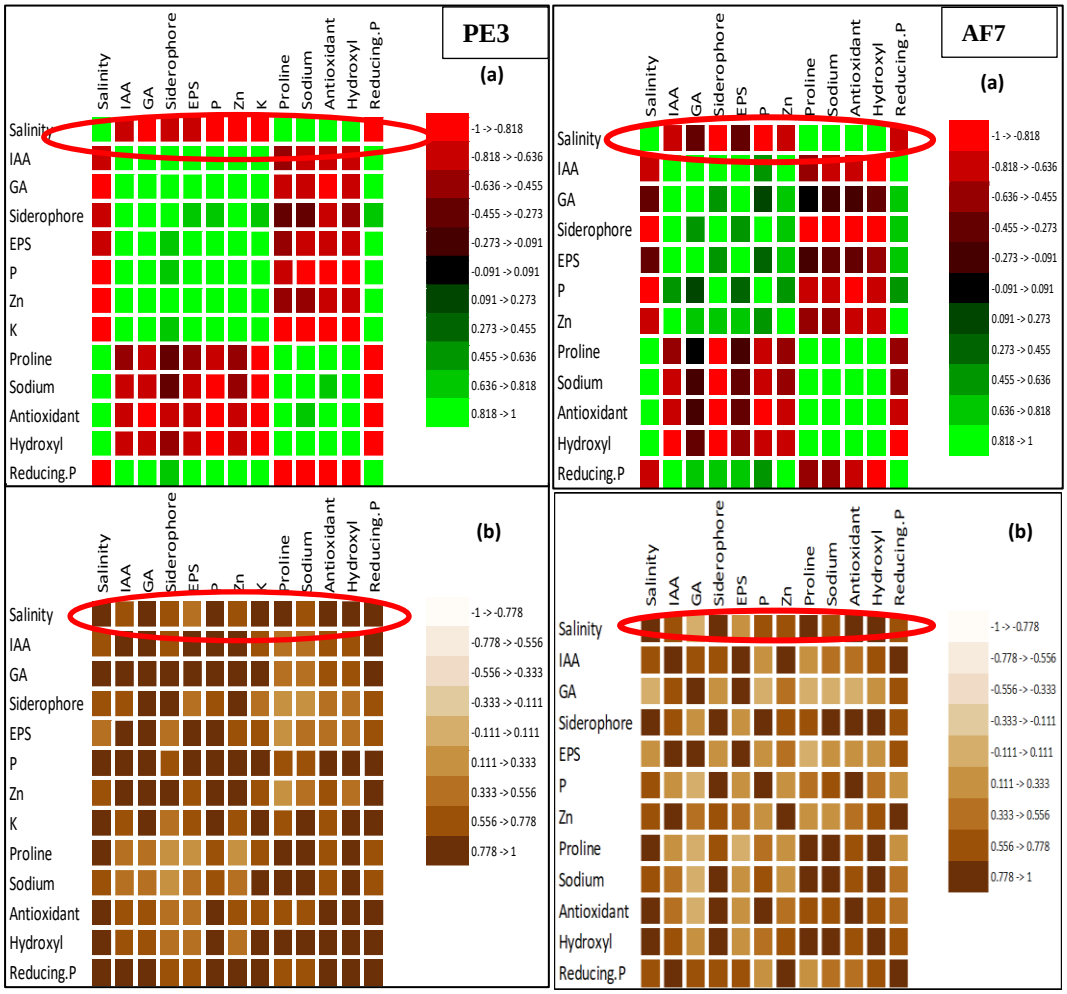
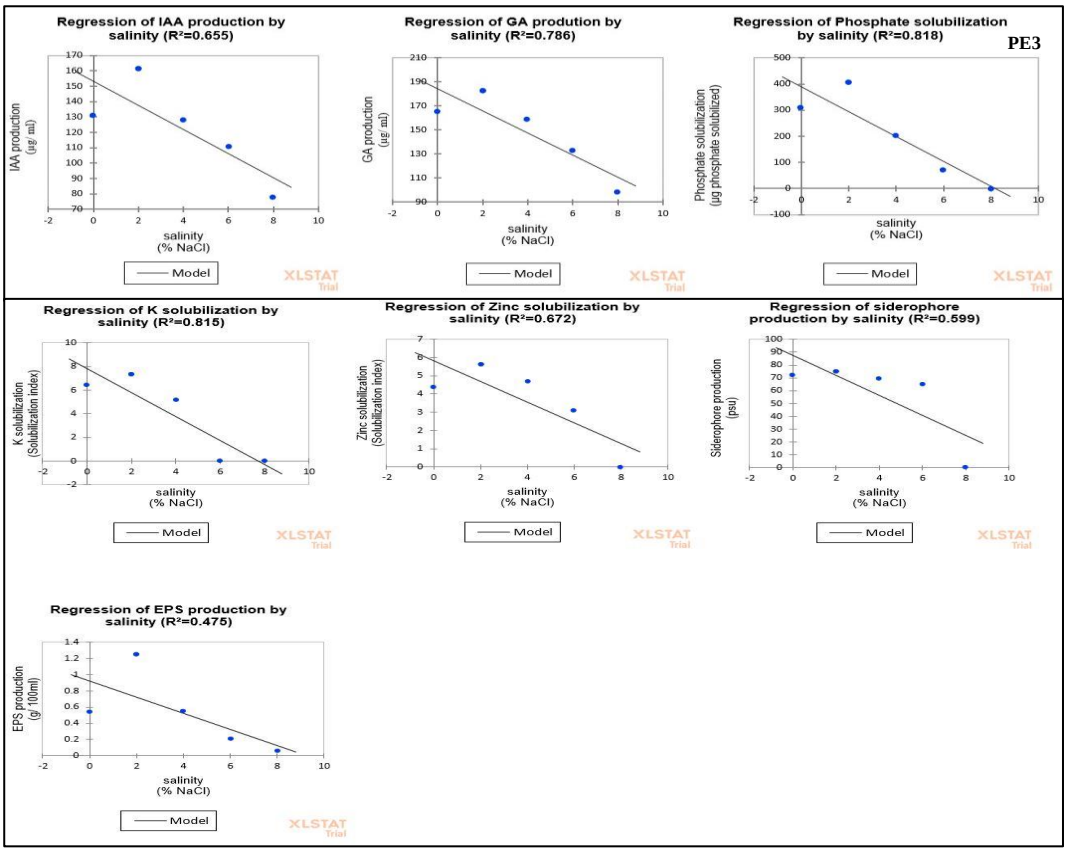
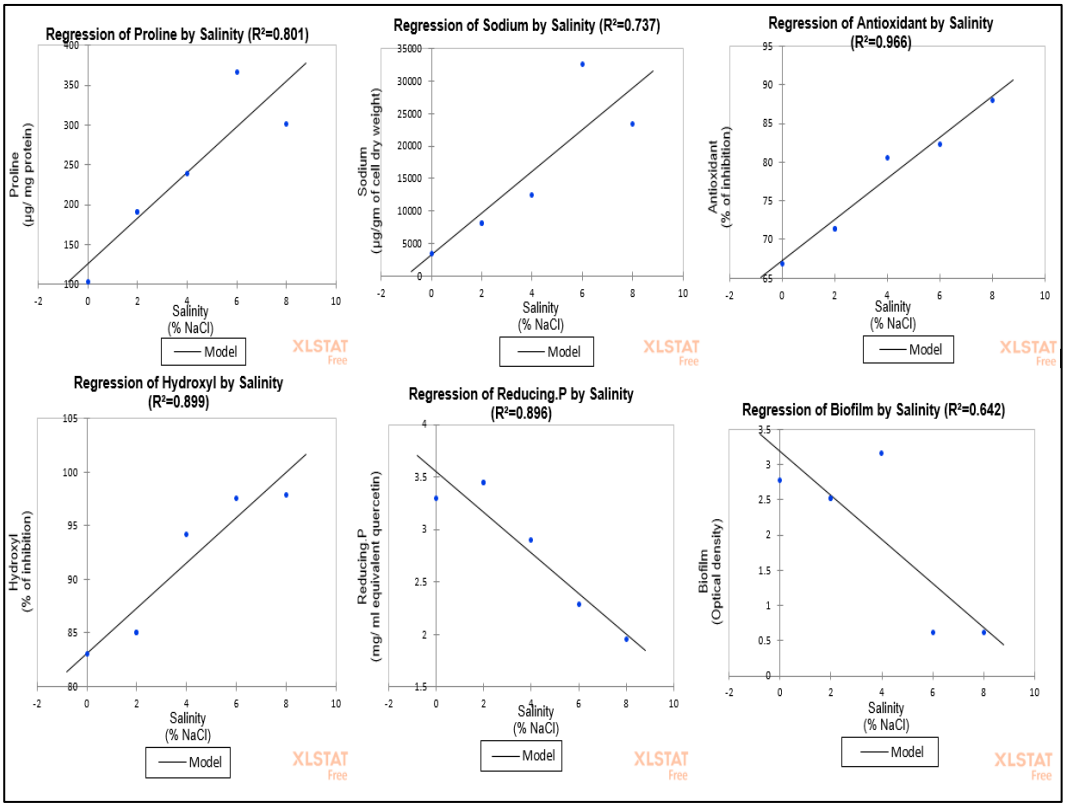


Fig. 14 Based on Pearson's correlation of coefficient (*P < 0.05)* determining the relationship between salinity and plant growth promoting and salt tolerance traits of AF7 and PE3 **(a) Image of the correlation matrix (b) Image of the matrix of coefficients of determination (R^2)**

IAA- IAA production, GA- Gibberellic acid production, Siderophore- Siderophore production, EPS-exopolysaccharide production, P- Phosphate solubilization, Zn- zinc solubilization, Proline- Proline accumulation, Sodium- Sodium accumulation, Antioxidant- Antioxidant activity, Hydroxyl- Hydroxyl scavenging activity, Reducing.P- Reducing power


Regression of IAA production by salinity (R²=0.655)
IAA production (µg/ ml)
salinity (% NaCl)
Model
Regression of GA prodution by salinity (R²=0.786)
GA production (µg/ ml)
salinity (% NaCl)
Model
Regression of Phosphate solubilization by salinity (R²=0.818)
PE3
Phosphate solubilization (µg phosphate solubilized)
salinity (% NaCl)
Model
Regression of K solubilization by salinity (R²=0.815)
K solubilization (Solubilization index)
salinity (% NaCl)
Model
Regression of Zinc solubilization by salinity (R²=0.672)
Zinc solubilization (Solubilization index)
salinity (% NaCl)
Model
Regression of siderophore production by salinity (R²=0.599)
Siderophore production (psu)
salinity (% NaCl)
Model
Regression of EPS production by salinity (R²=0.475)
EPS production (g/100ml)
salinity (% NaCl)
Model
XLSTAT Trial


Regression of Proline by Salinity (R²=0.801)
Proline (µg/ mg protein)
Salinity (% NaCl)
Model
Regression of Sodium by Salinity (R²=0.737)
Sodium (µg/gm of cell dry weight)
Salinity (% NaCl)
Model
Regression of Antioxidant by Salinity (R²=0.966)
Antioxidant (% of inhibition)
Salinity (% NaCl)
Model
Regression of Hydroxyl by Salinity (R²=0.899)
Hydroxyl (% of inhibition)
Salinity (% NaCl)
Model
Regression of Reducing.P by Salinity (R²=0.896)
Reducing P (mg/ ml equivalent quercetin)
Salinity (% NaCl)
Model
Regression of Biofilm by Salinity (R²=0.642)
Biofilm (Optical density)
Salinity (% NaCl)
Model
XLSTAT Free

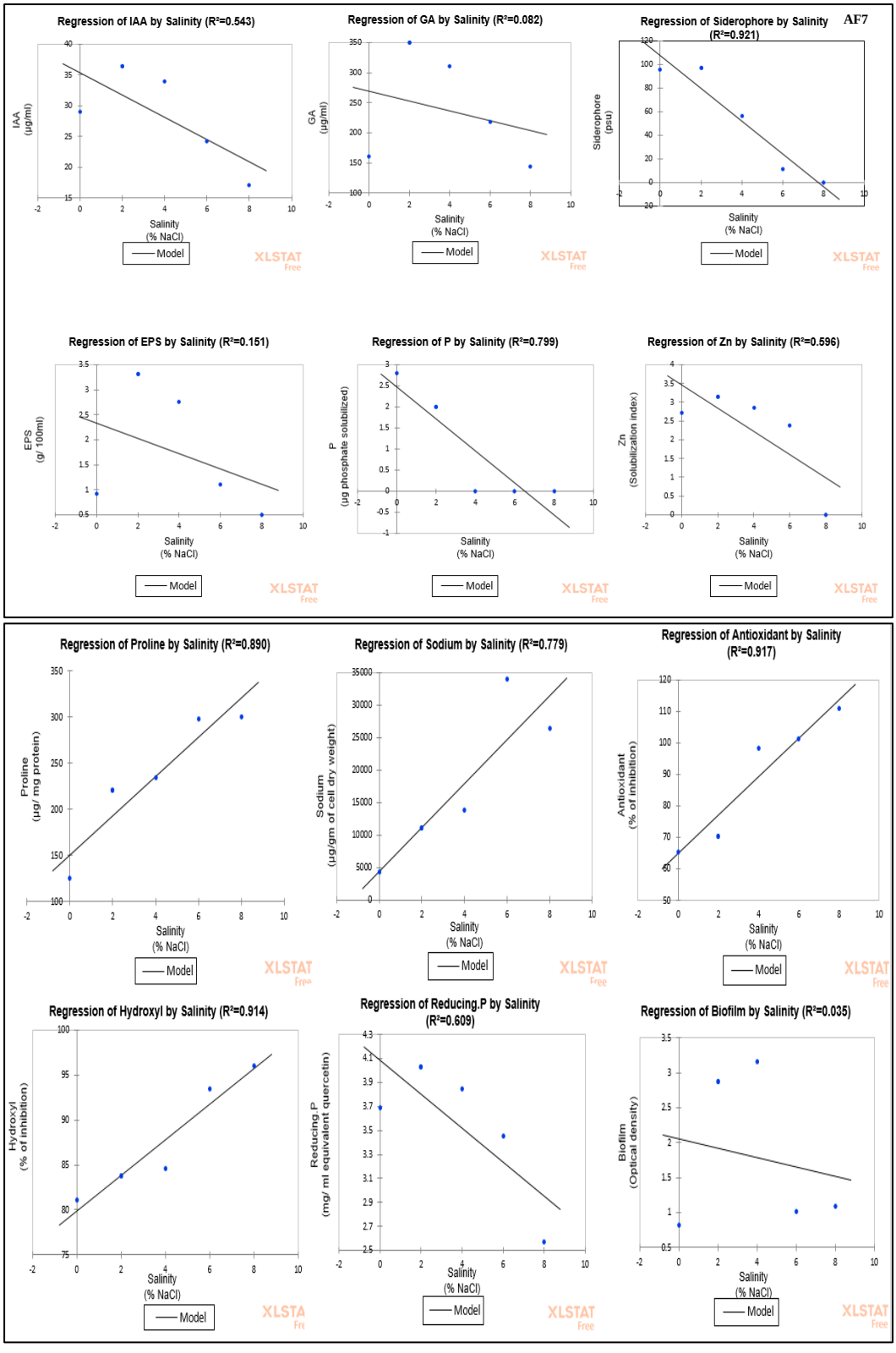
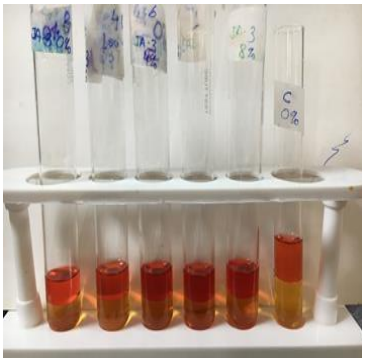
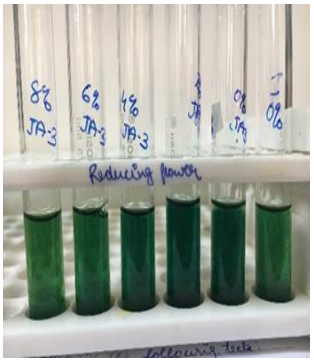


Fig. 15 Linear regression analysis showing effect of increasing salinity (from control to 8% NaCl) on plant growth promoting properties of PE3 and AF7.
Points on the trendline represent mean values of PGP properties (n= 3) and prediction of the model is represented by R^2 value (significance level $P < 0.05$)

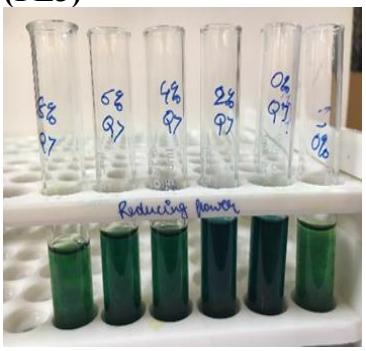


Proline accumulation (PE3)

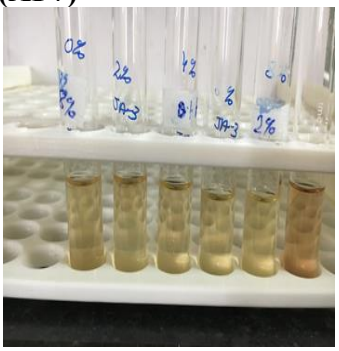
Proline accumulation (AF7)



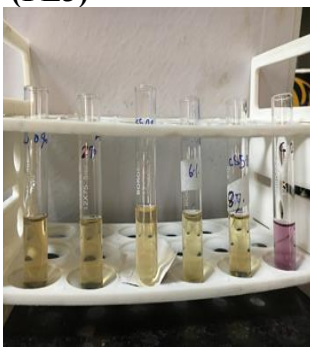
Reducing power (PE3)



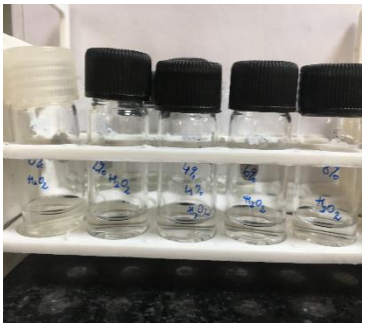
Reducing power (AF7)



Antioxidant activity (DPPH assay) (PE3)



Antioxidant activity (DPPH assay) (AF7)



Hydroxyl scavenging activity (PE3)

Hydroxyl scavenging activity (AF7)

Sodium accumulation (Flame photometry)

Fig. 16 Estimation of various salt-tolerance properties of selected isolates (PE3 and AF7) under different saline conditions (2%-8% NaCl) and non-saline control

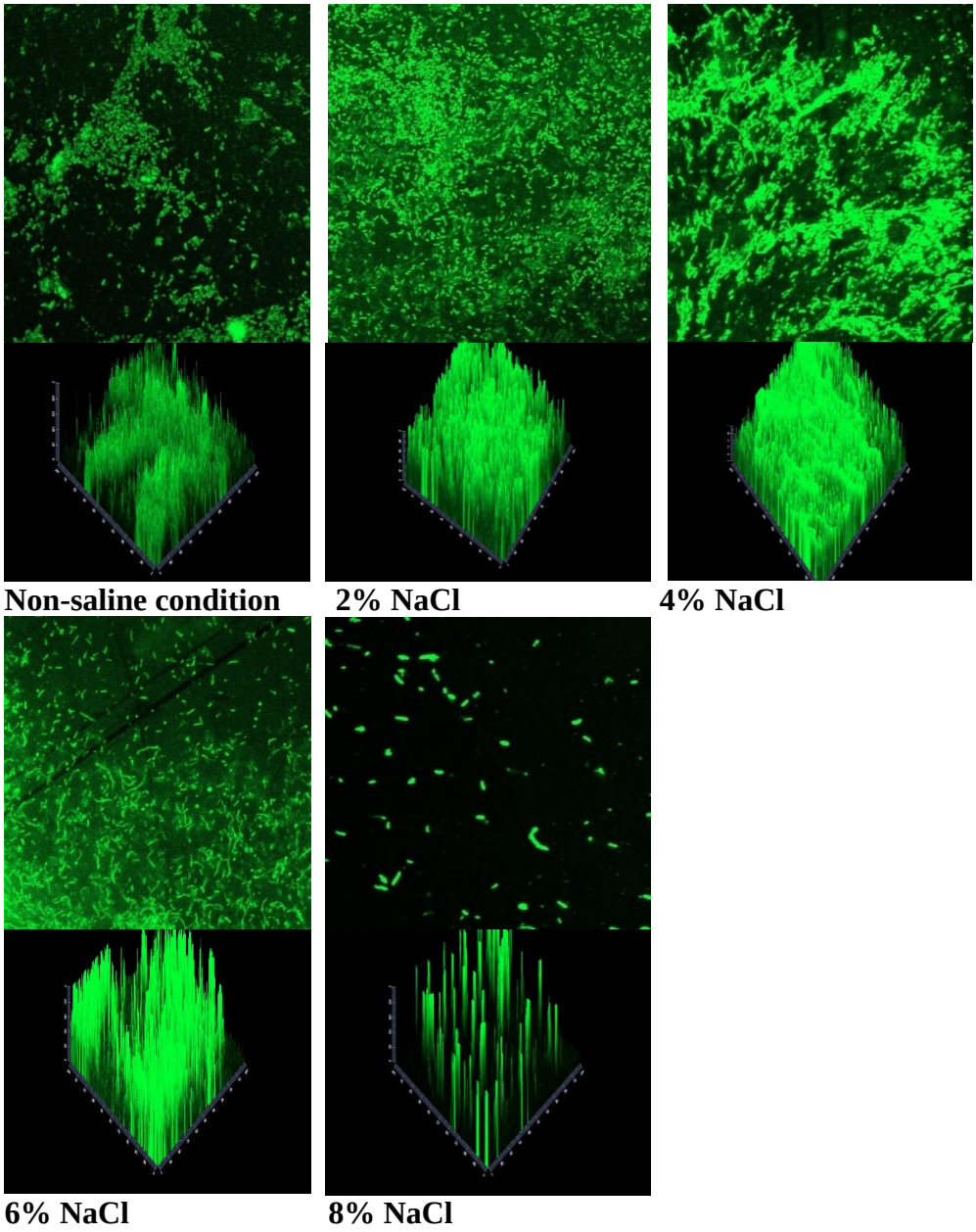


Fig. 17 ILSCM analysis of Acridine orange stained PE3 biofilms under different saline conditions. The figure shows 2D and 2.5D Z-stacks of each developed biofilms on glass surface

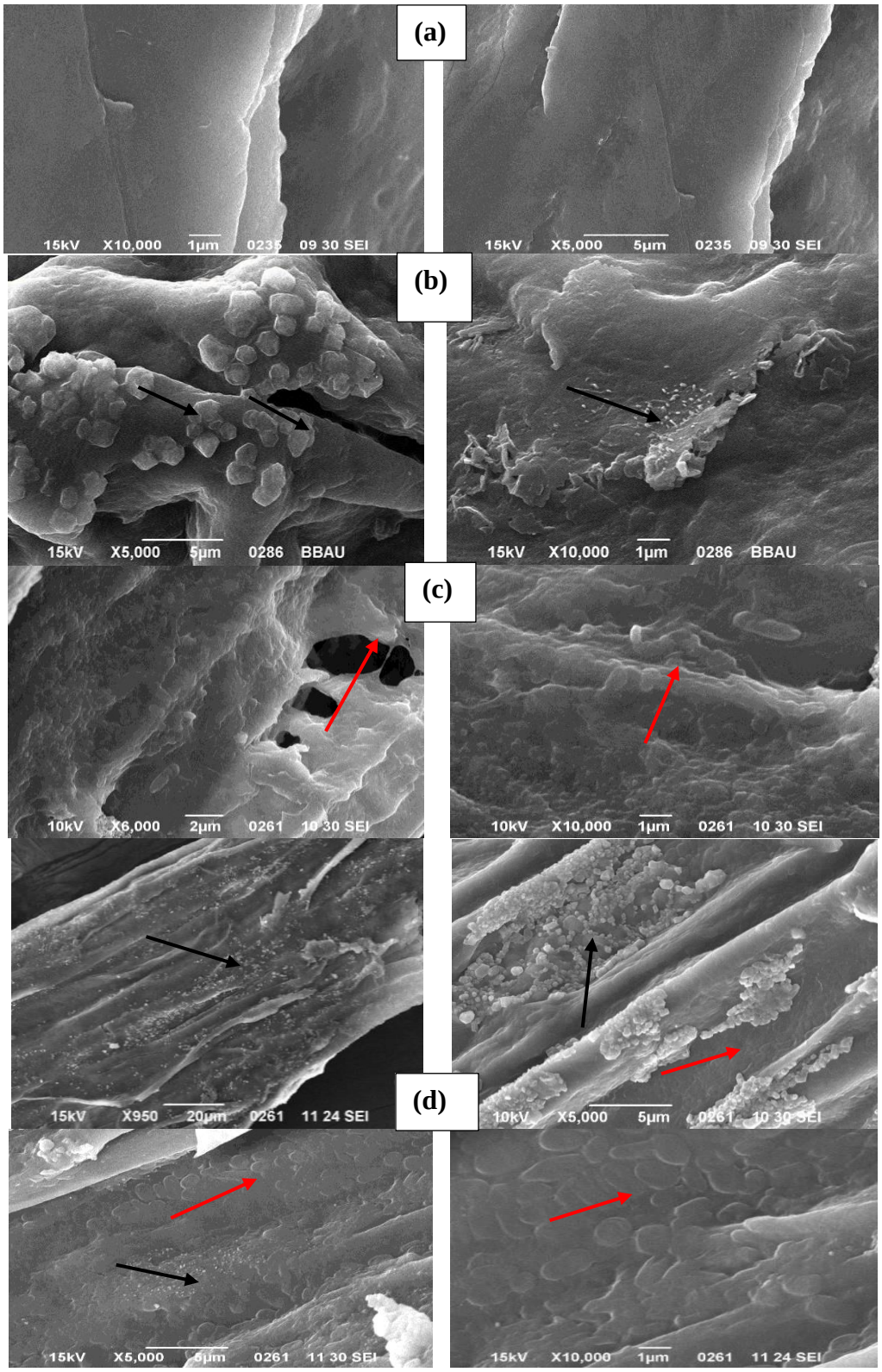


Fig. 18 Formation of biofilm on root surface using different treatments: **(a)** untreated control roots **(b)** 2% EPS **(c)** PE3 only, **(d)** PE3 plus 2% EPS, analyzed through SEM under various magnification.
Black arrow demarcates the presence of sugar crystals and red arrow shows PE3 macro-colonies inside mucilaginous biofilm

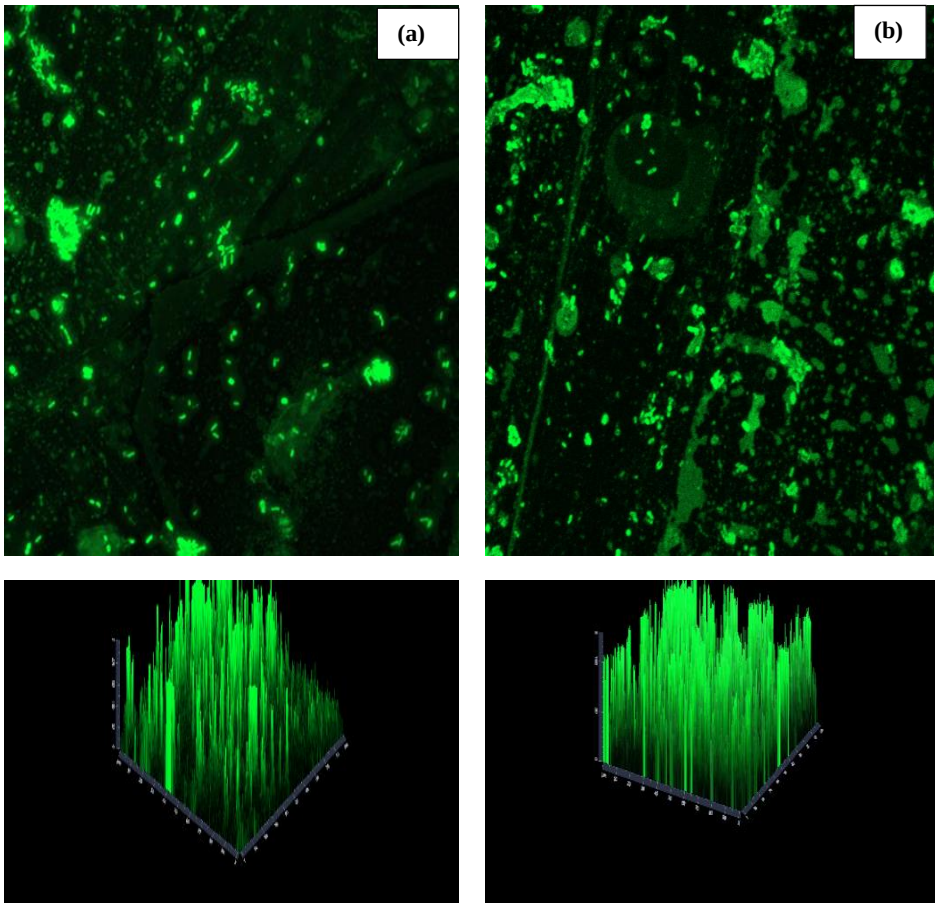


Fig. 19 ILSCM analysis of biofilm formation on sunflower root surface when treated with **(a)** PE3 alone **(b)** 2% EPS plus PE3

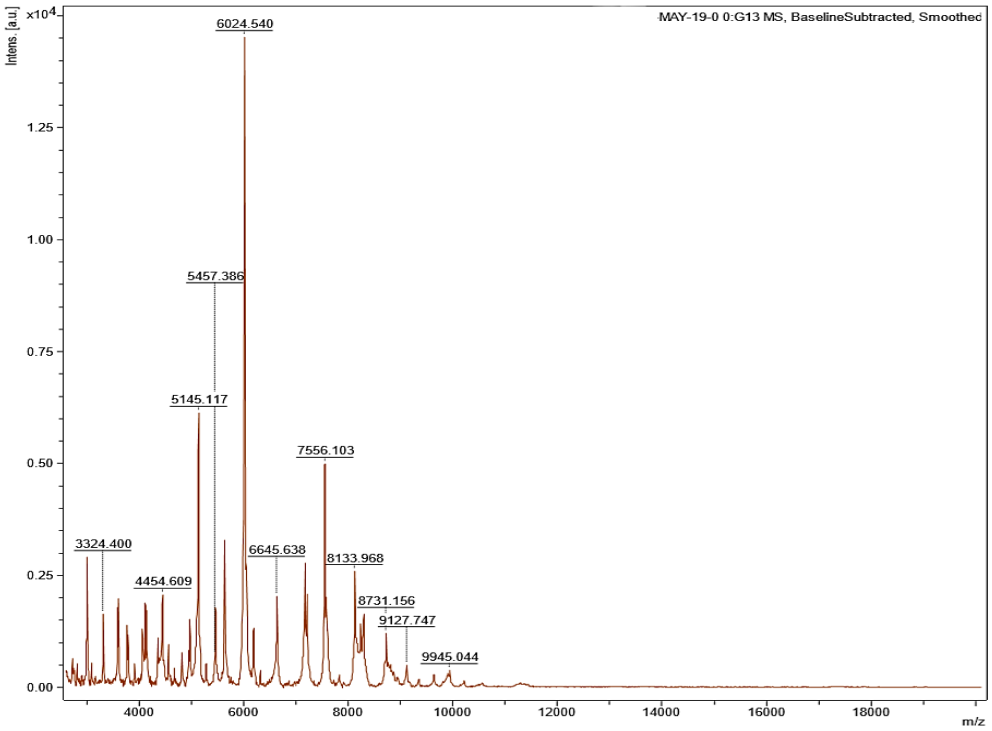


Fig. 20 MALDI-TOF MS spectra of bacterial isolate PE3 indicating the protein profile (2-20KDa)

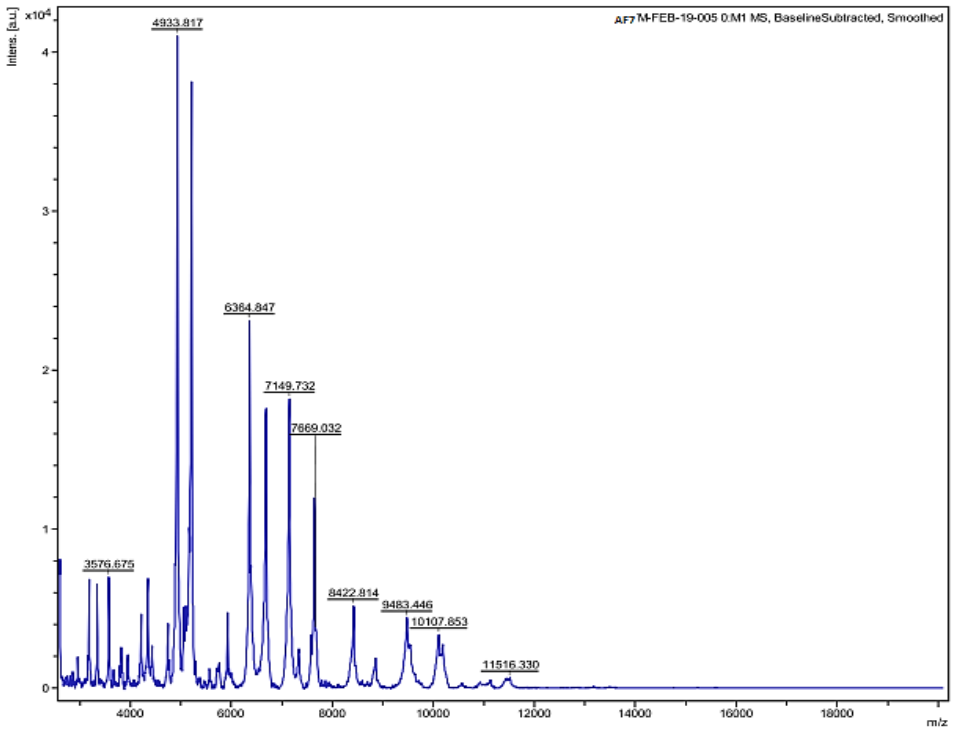


Fig. 21 MALDI-TOF MS spectra of bacterial isolate AF7 indicating the protein profile (2-20KDa)

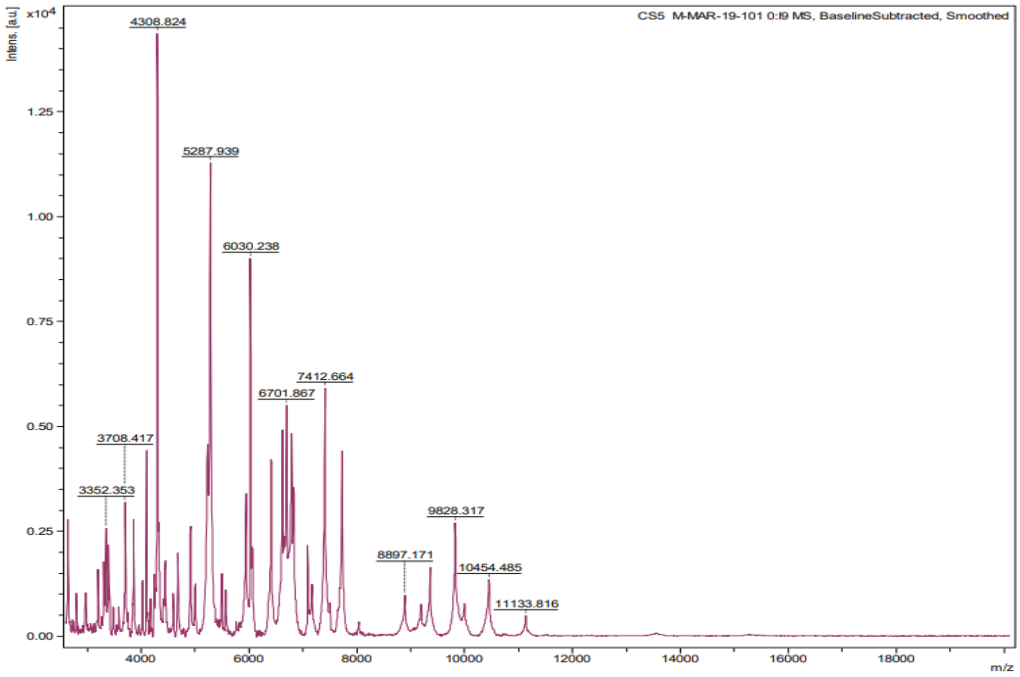


Fig. 22 MALDI-TOF MS spectra of bacterial isolate CS5 indicating the protein profile (2-20KDa)

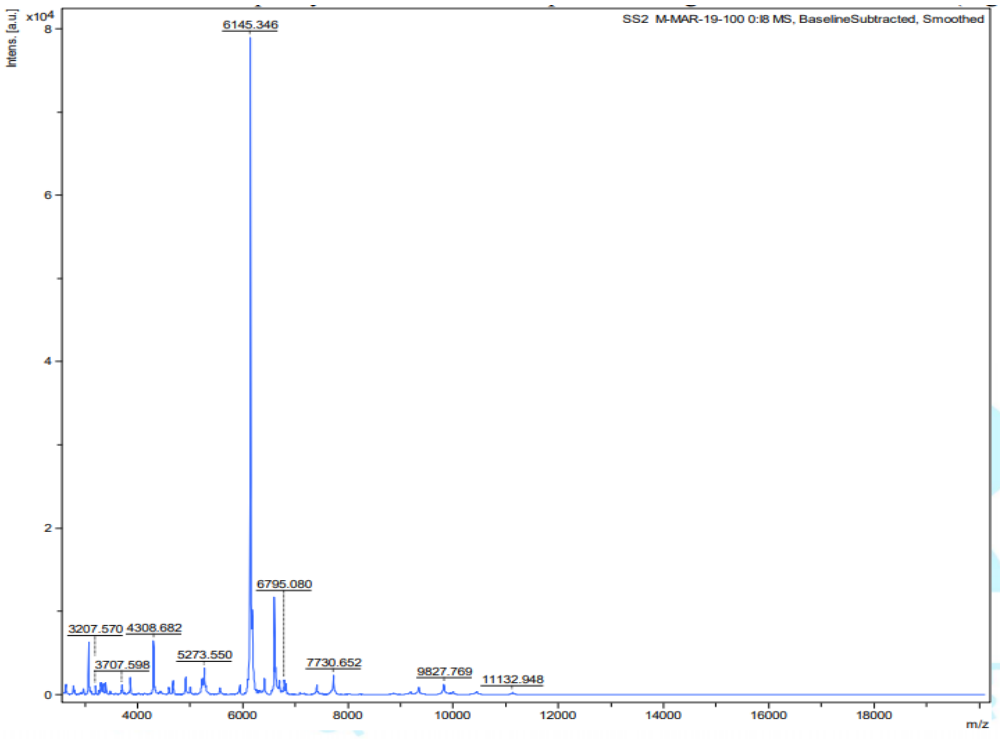


Fig. 23 MALDI-TOF MS spectra of bacterial isolate SS2 indicating the protein profile (2-20KDa)

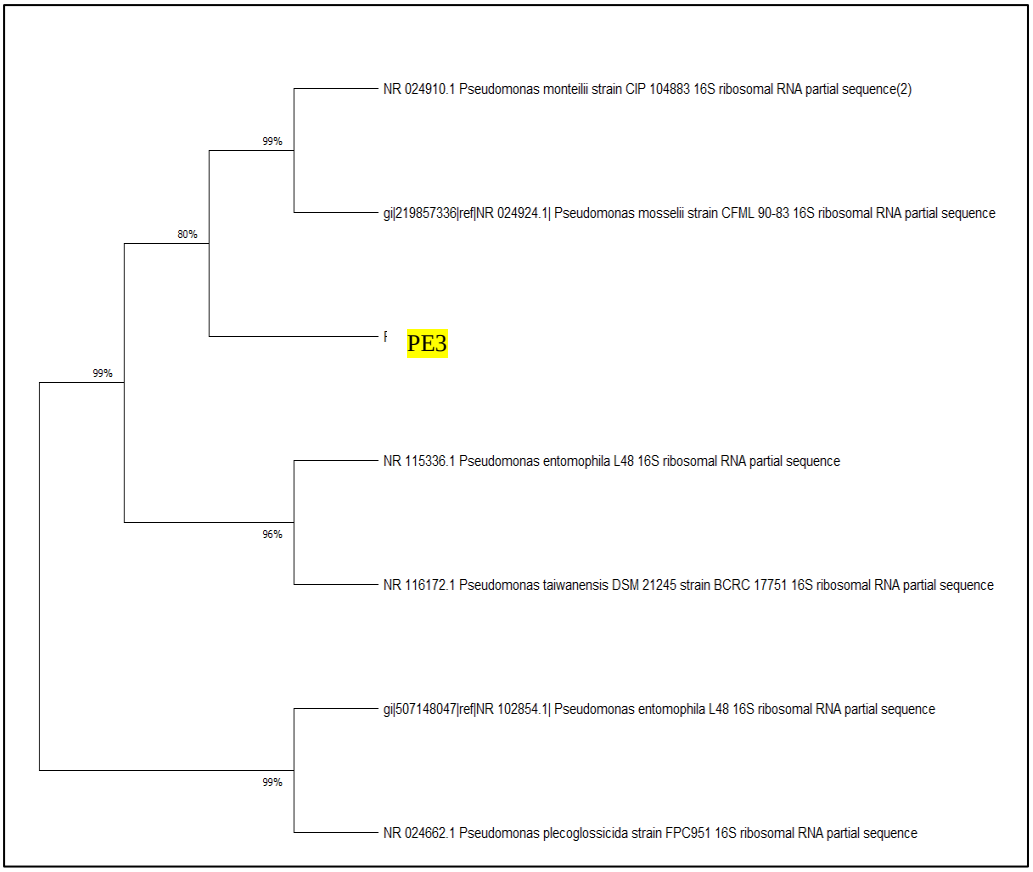


Fig. 24 Phylogenetic tree analysis of isolate PE3

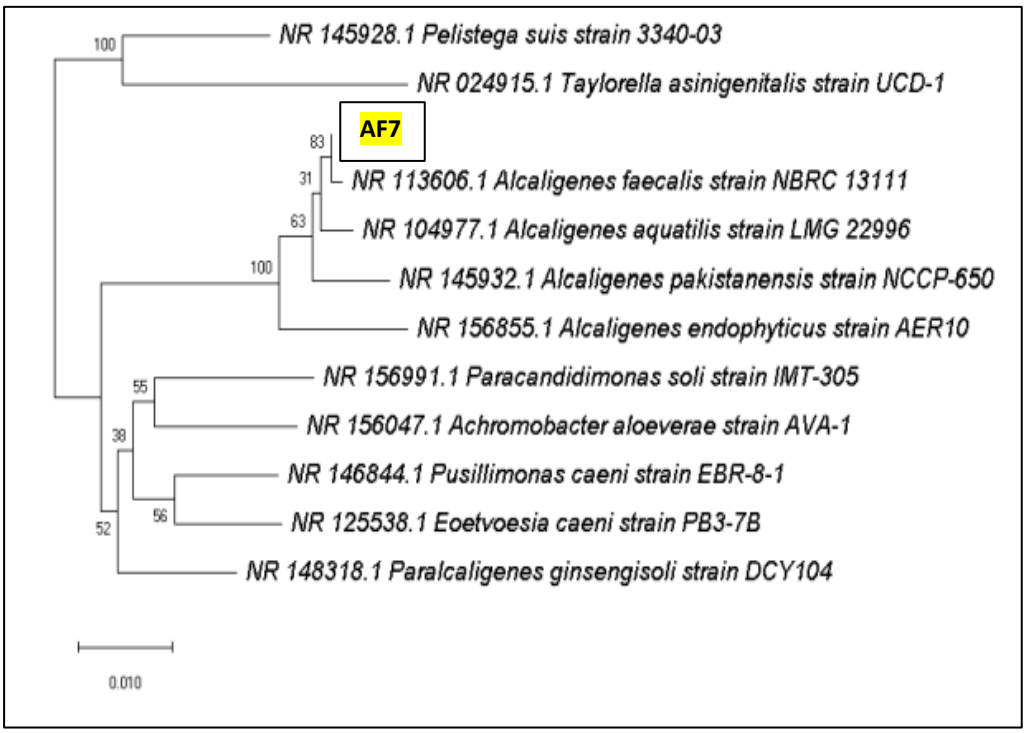


Fig. 25 Phylogenetic tree analysis of isolate AF7

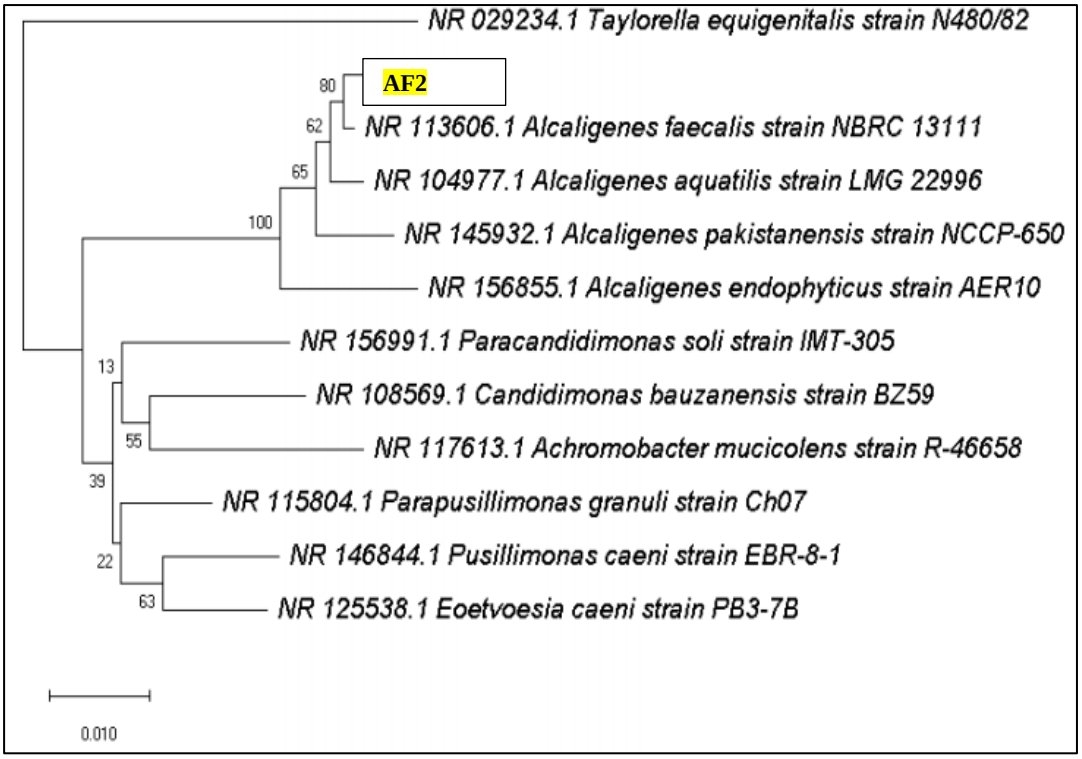


Fig. 26 Phylogenetic tree analysis of AF2

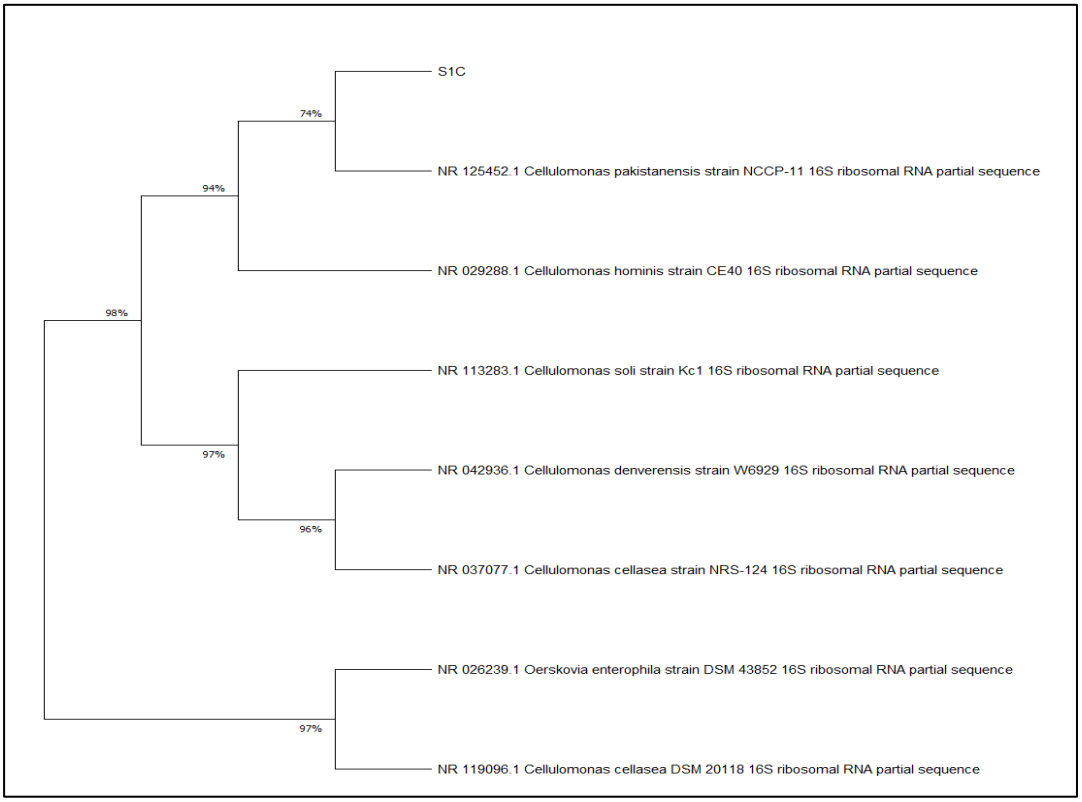


Fig 27 Phylogenetic tree analysis of isolate S1C

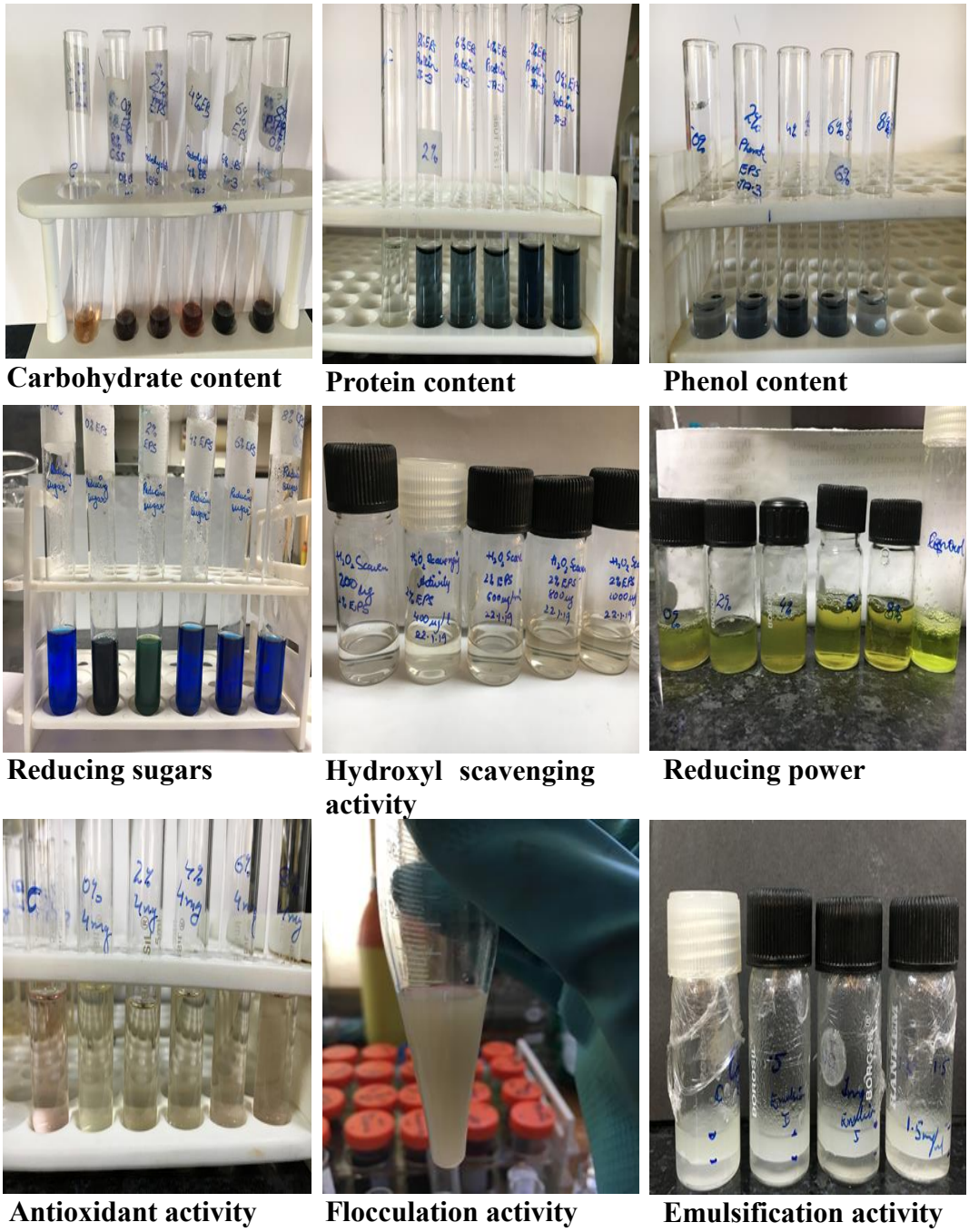
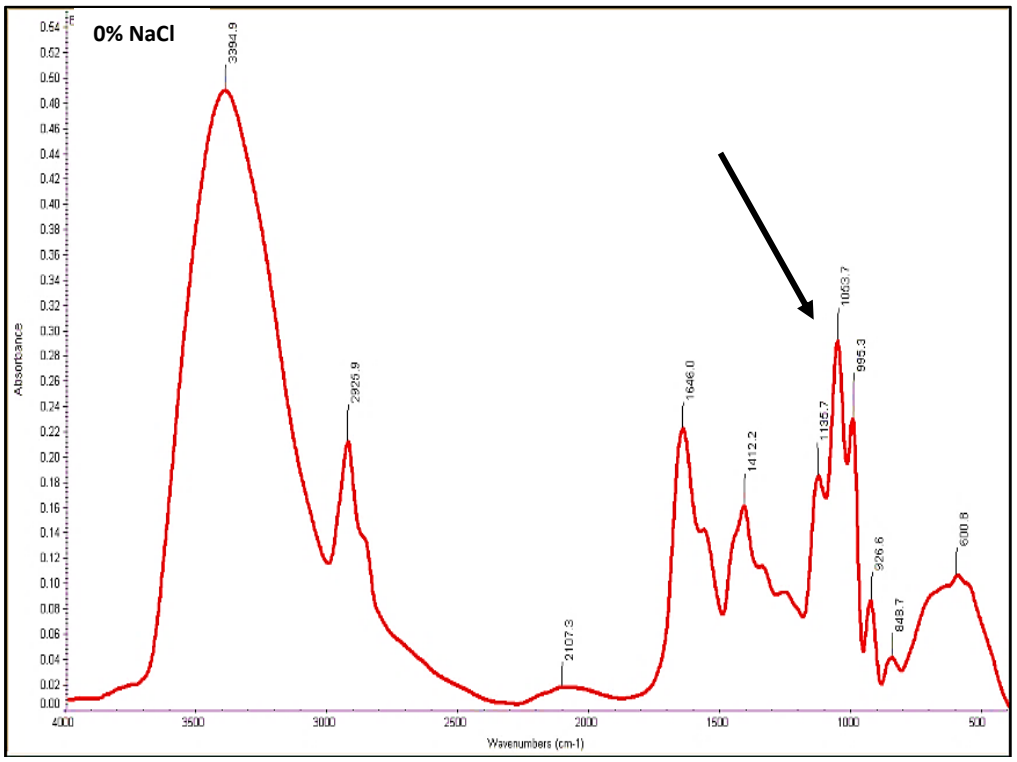
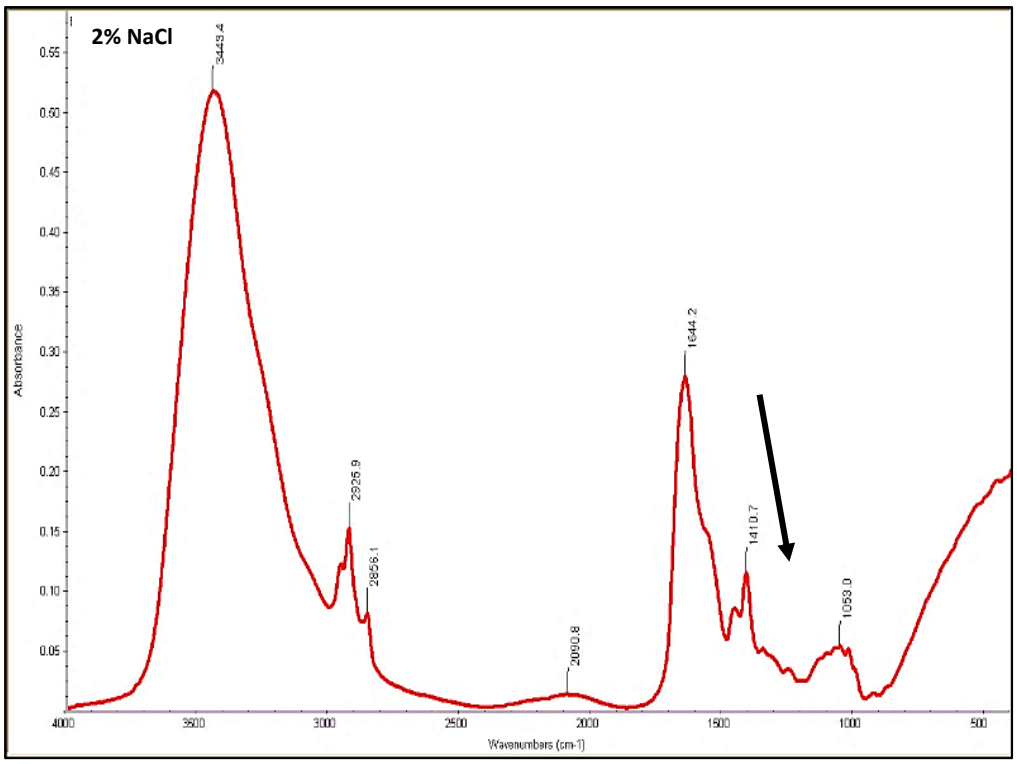
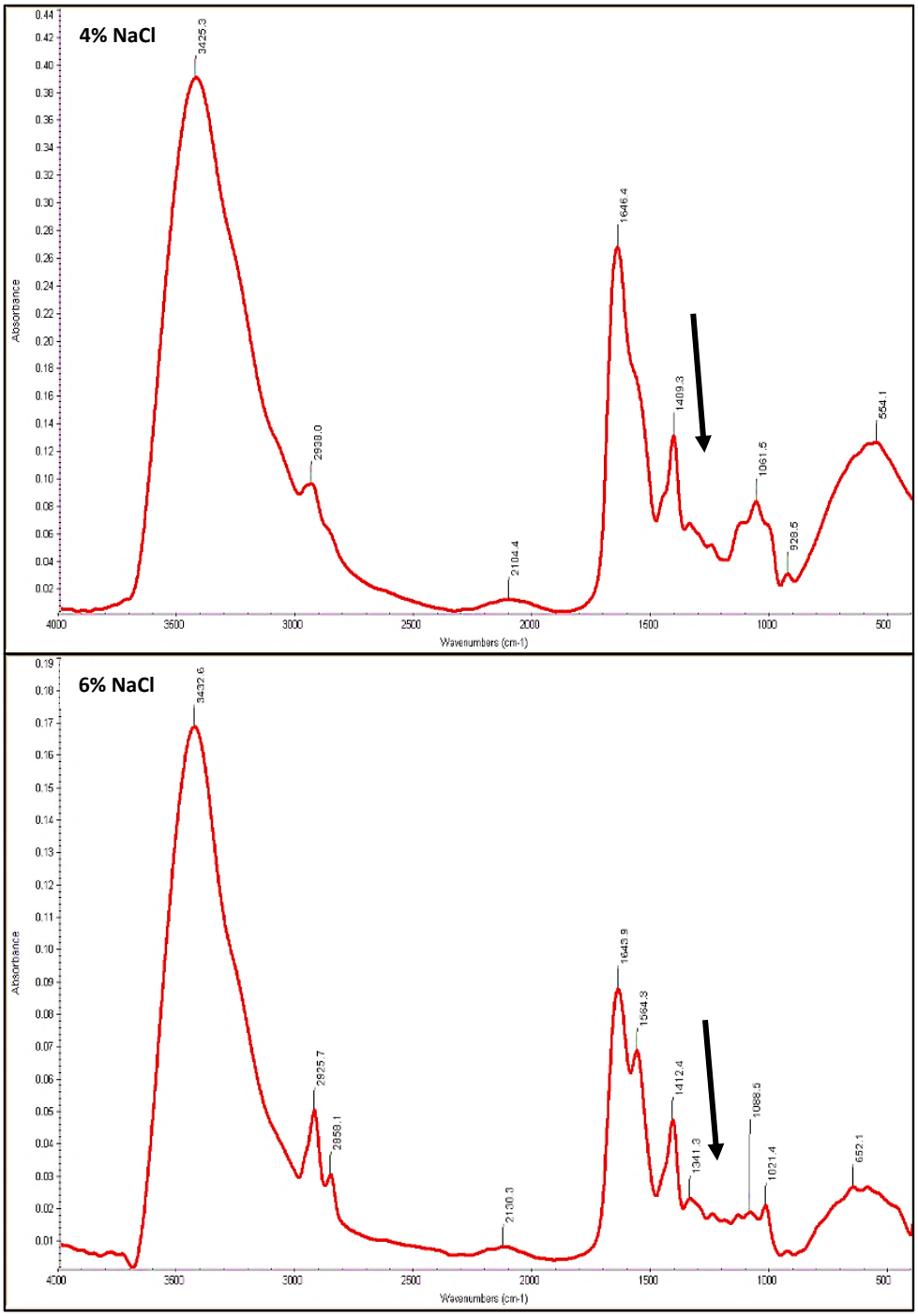


Fig. 28 Compositional analysis and salt-tolerance traits of PE3-extracted exopolysaccharides under various saline conditions


0% NaCl
3394.9
2925.9
2107.3
1646.0
1412.2
1135.7
1053.7
995.3
926.6
848.7
600.8
Absorbance
Wavenumbers (cm-1)


2% NaCl
3449.4
2926.9
2856.1
2090.8
1644.2
1410.7
1059.0
Absorbance
Wavenumbers (cm-1)


4% NaCl
3425.3
2938.0
2104.4
1646.4
1409.3
1061.5
928.5
554.1
Absorbance
Wavenumbers (cm-1)
6% NaCl
3432.6
2925.7
2859.1
2130.3
1643.9
1564.3
1412.4
1341.3
1088.5
1021.4
652.1
Absorbance
Wavenumbers (cm-1)

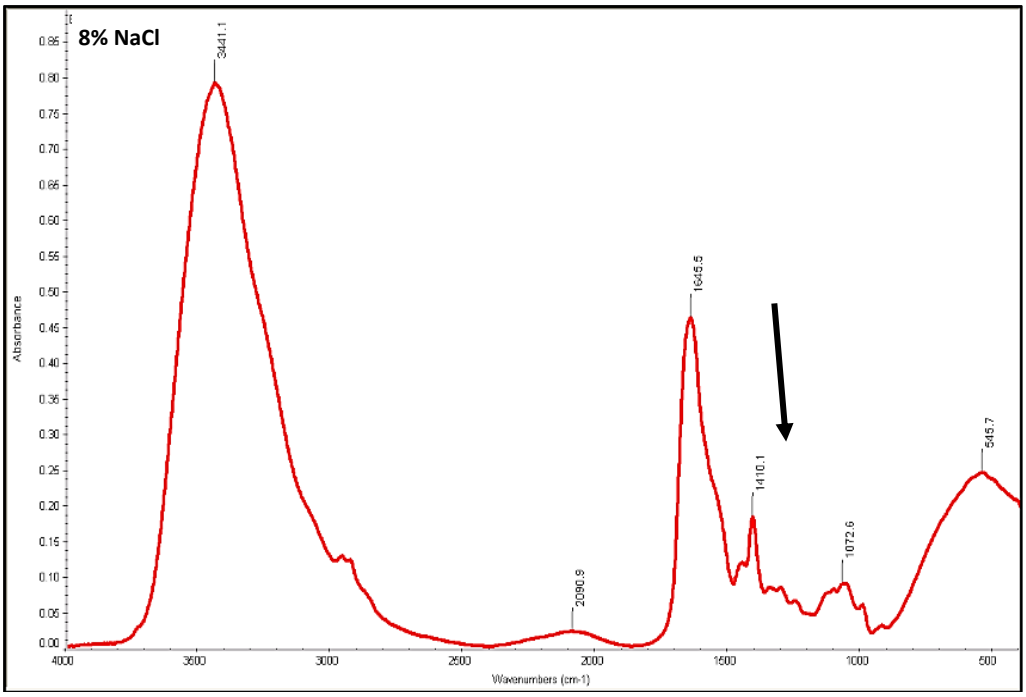
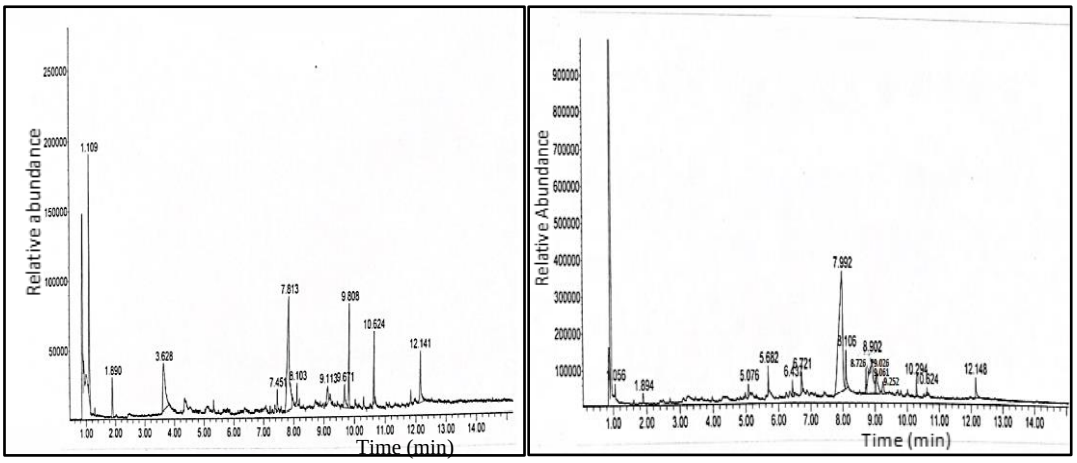
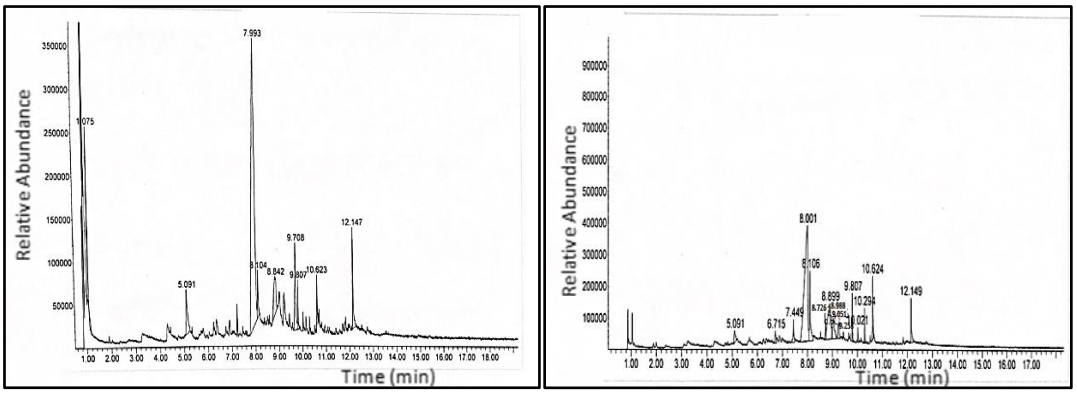


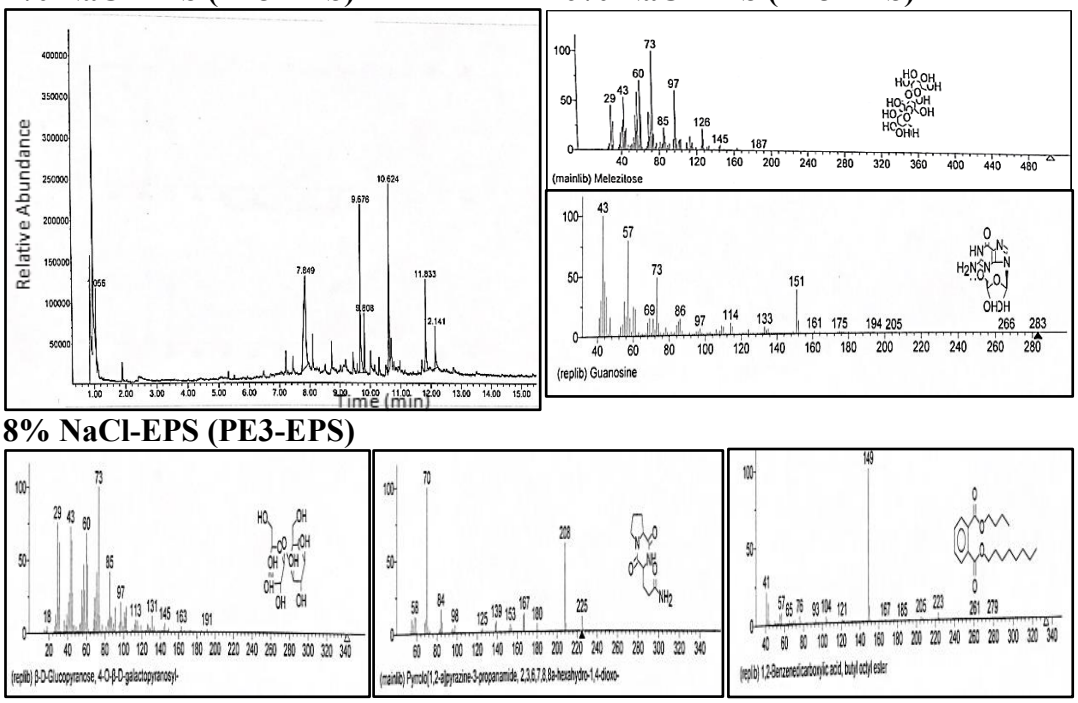
Fig. 29 Effect of salt stress on functional groups and bonding of EPS (produced by isolate PE3) through FT-IR analysis.
Arrow demarcates the major modification in peaks reported on exposure to salinity



Non-salinized EPS (PE3-EPS) **2% NaCl-EPS (PE3-EPS)**

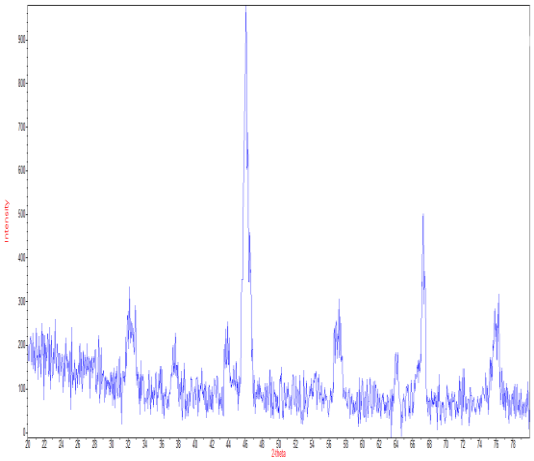


4% NaCl-EPS (PE3-EPS) **6% NaCl-EPS (PE3-EPS)**

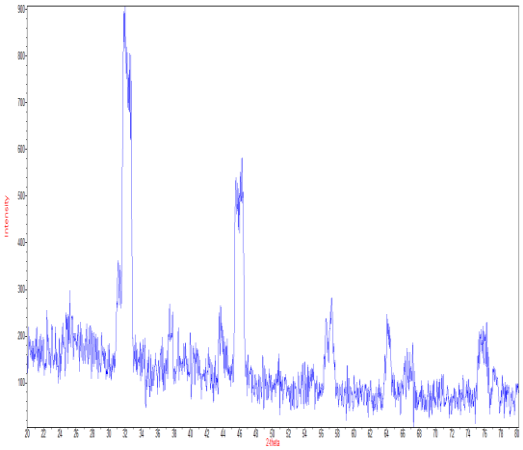


8% NaCl-EPS (PE3-EPS)

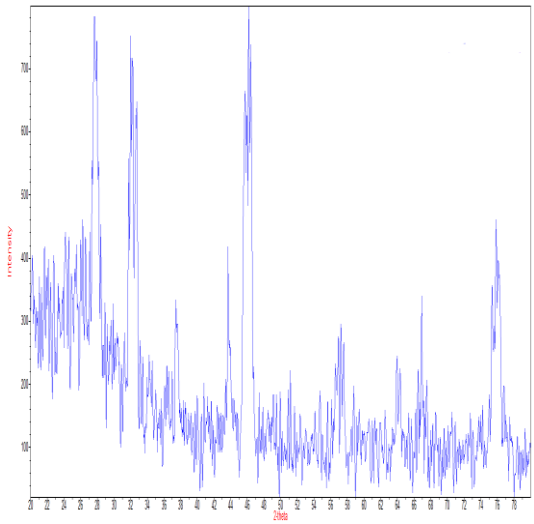
Fig. 30 Detection of monomeric-unit and composition of PE3 extracted EPS under various saline conditions through GC-MS analysis



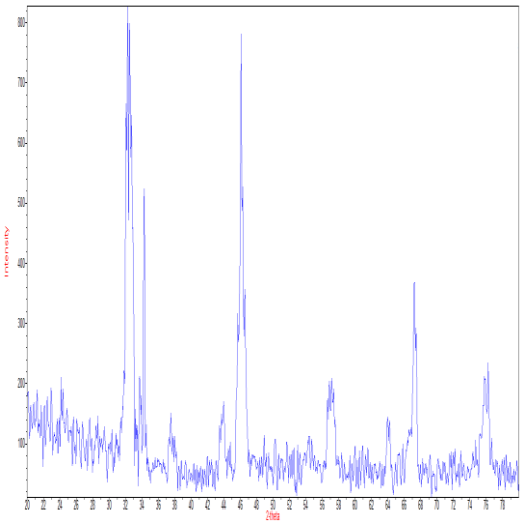
Non-saline control PE3-EPS



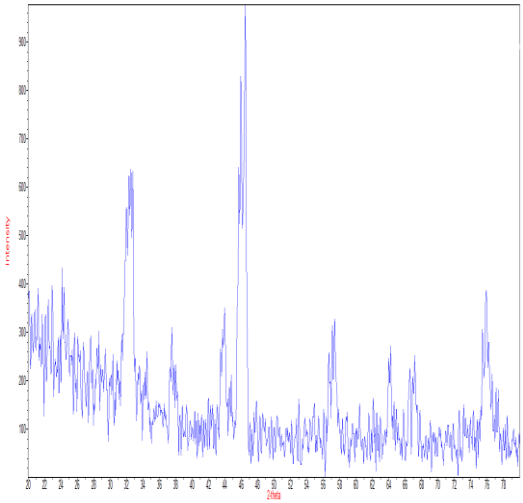
2% NaCl PE3-EPS



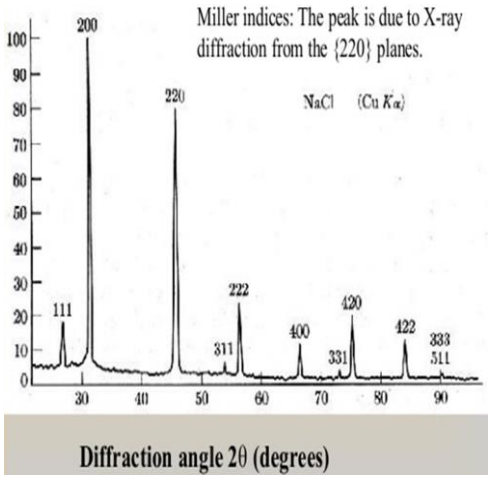
4% NaCl PE3-EPS



6% NaCl-EPS



8%NaCl-EPS



XRD analysis of NaCl (JCPDS No: 5-0628)

Fig. 31 XRD analysis of PE3-EPS extracted at various saline conditions and comparison with NaCl standard diffractogram, depicting changes in structure due to salinity

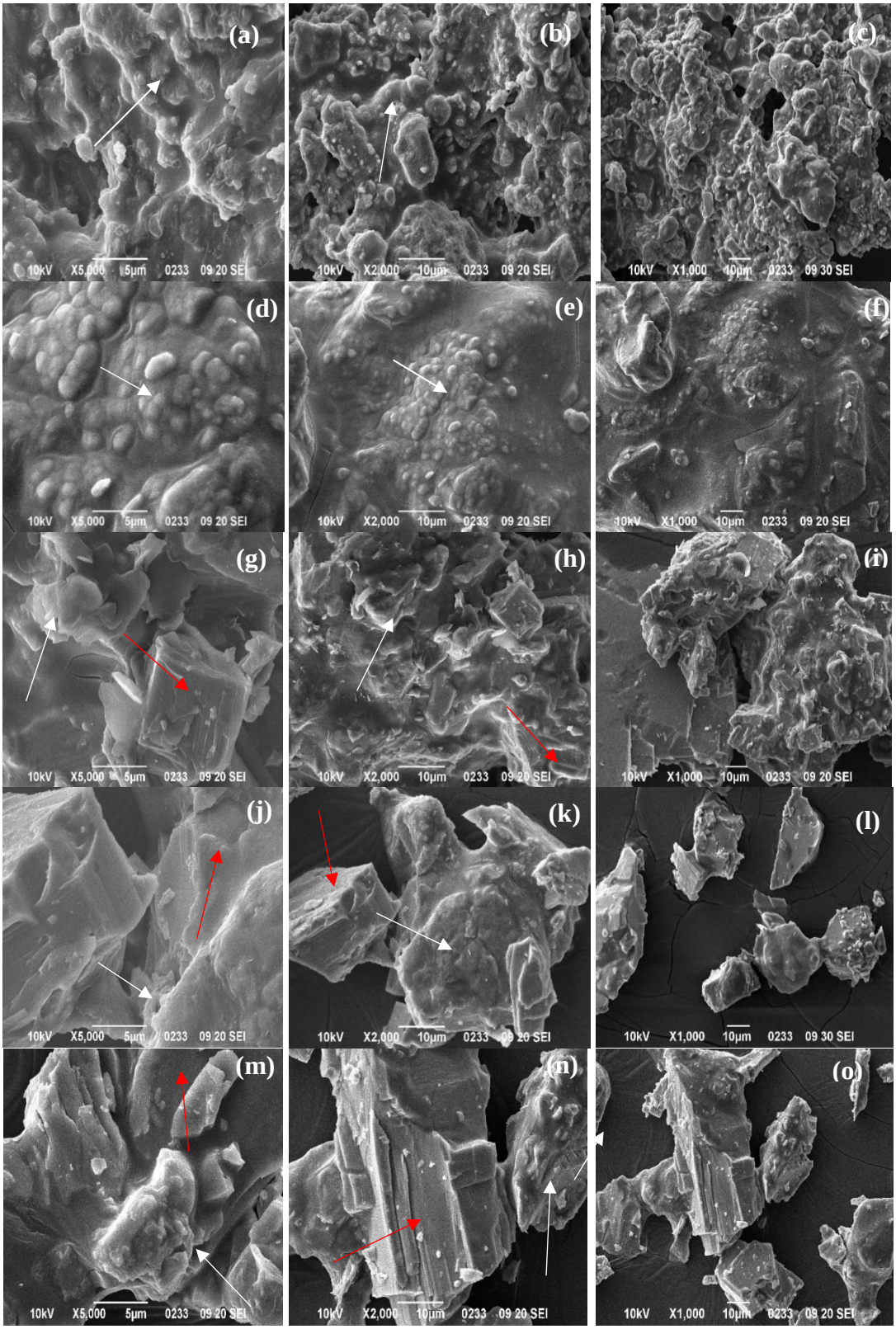


Fig. 32 SEM analysis of EPS produced by *P. entomophila* PE3 under various saline conditions. The change in morphology of EPS according to various salt concentrations are indicated as: **(a-c)** under non-saline conditions, **(d-f)** at 2% NaCl, **(g-i)** at 4% NaCl, **(j-l)** at 6% NaCl, **(n-o)** at 8% NaCl
Red and white arrow demarcates the presence of salt and sugar crystals in the EPS respectively

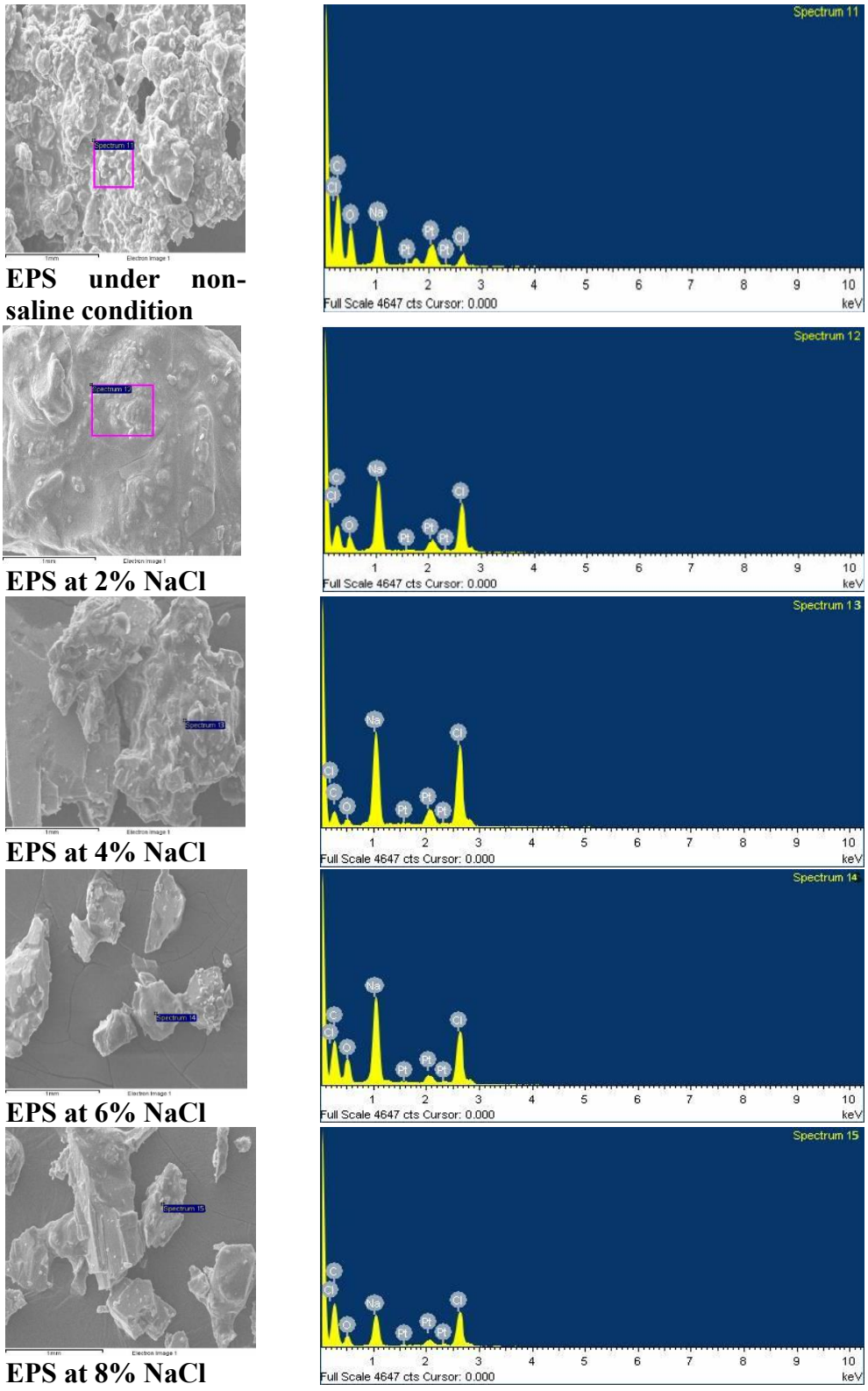
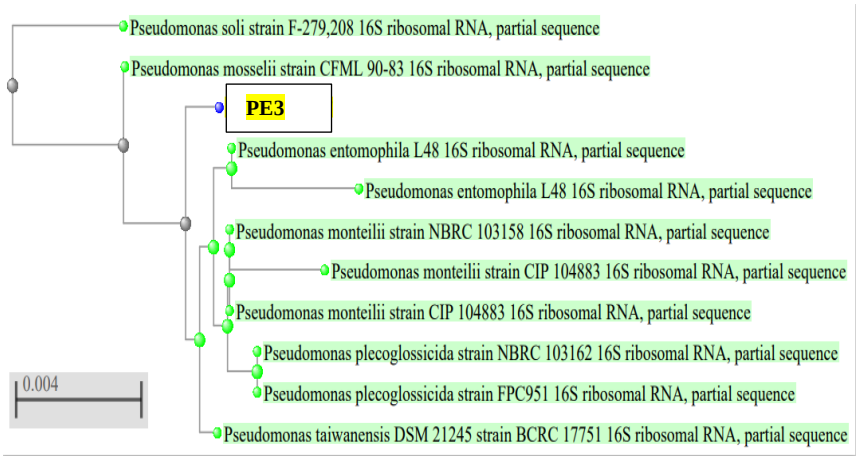
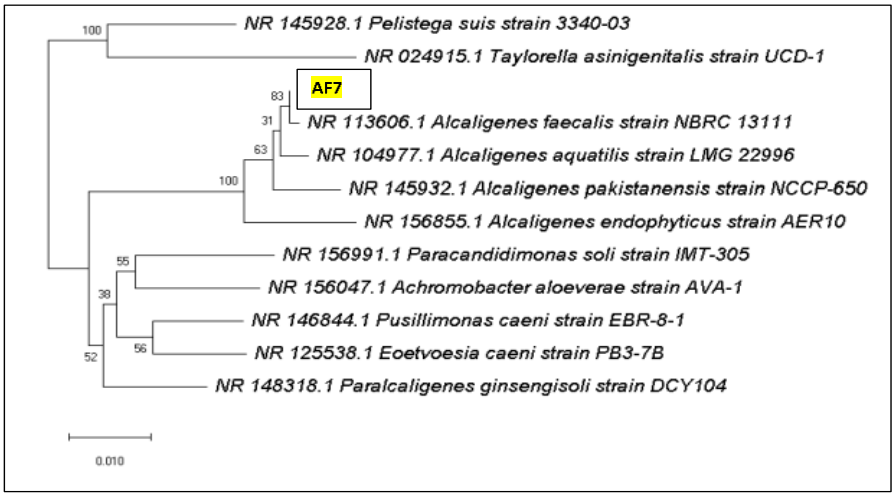


Fig. 33 SEM-EDS analysis illustrating the elementary composition of *P. entomophila* PE3 EPS extracted under various salt-concentrations



PE3



AF7

Fig. 34 Phylogenetic tree of isolates PE3 and AF7 isolated after colonization of roots in pot and field study

Fig. 35 Effect of various concentrations of EPS, isolates PE3 and AF7 (alone) and in combination with their respective EPS (2% EPS + bacteria), on growth of sunflower plants under saline conditions (pot study)

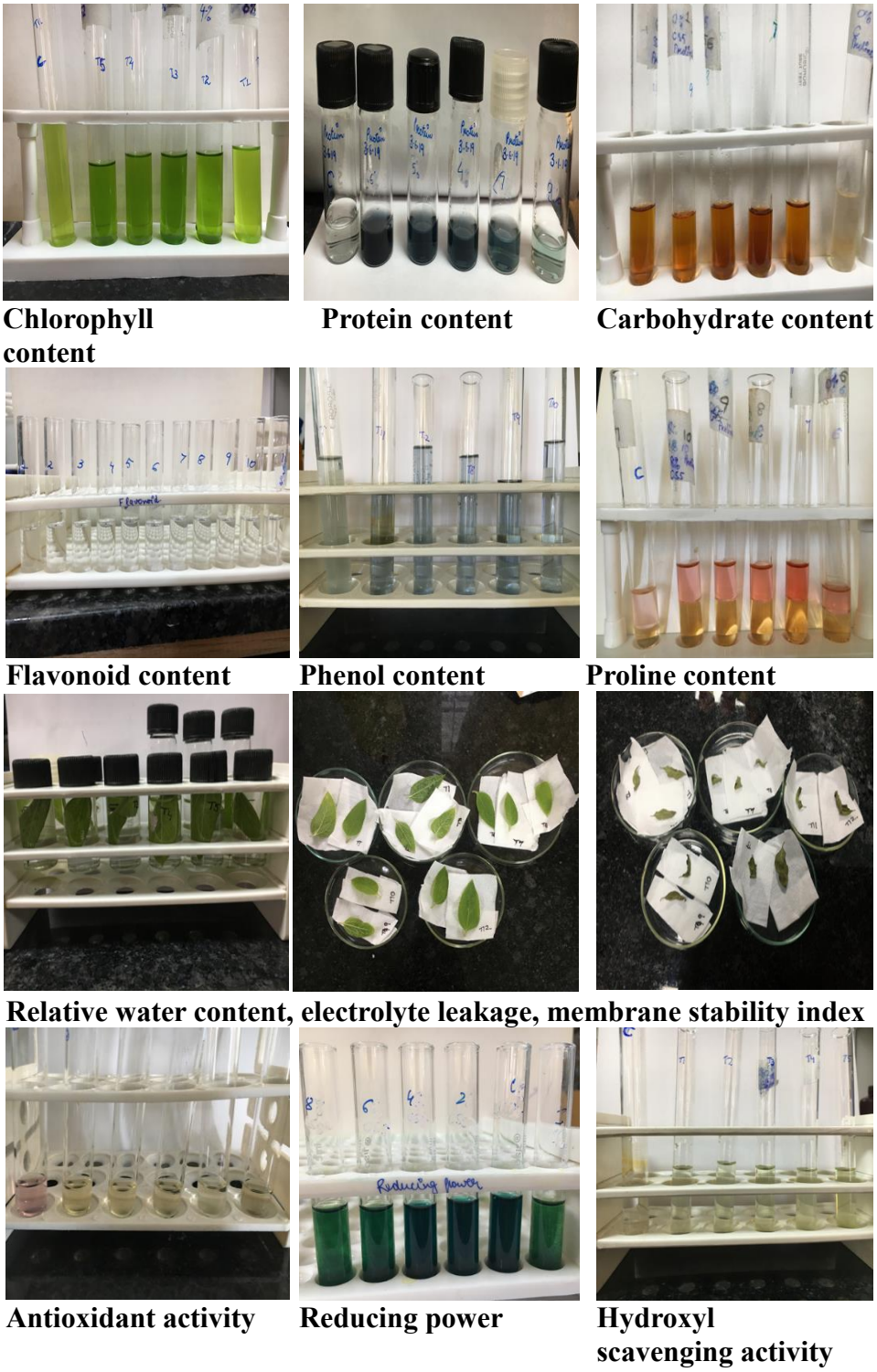


Fig. 36 Effect of various concentrations of EPS, isolates PE3 and AF7 (alone) and in combination with their respective EPS (2% EPS + bacteria), on biochemical and salt-tolerance properties of sunflower plants under saline conditions (pot study)

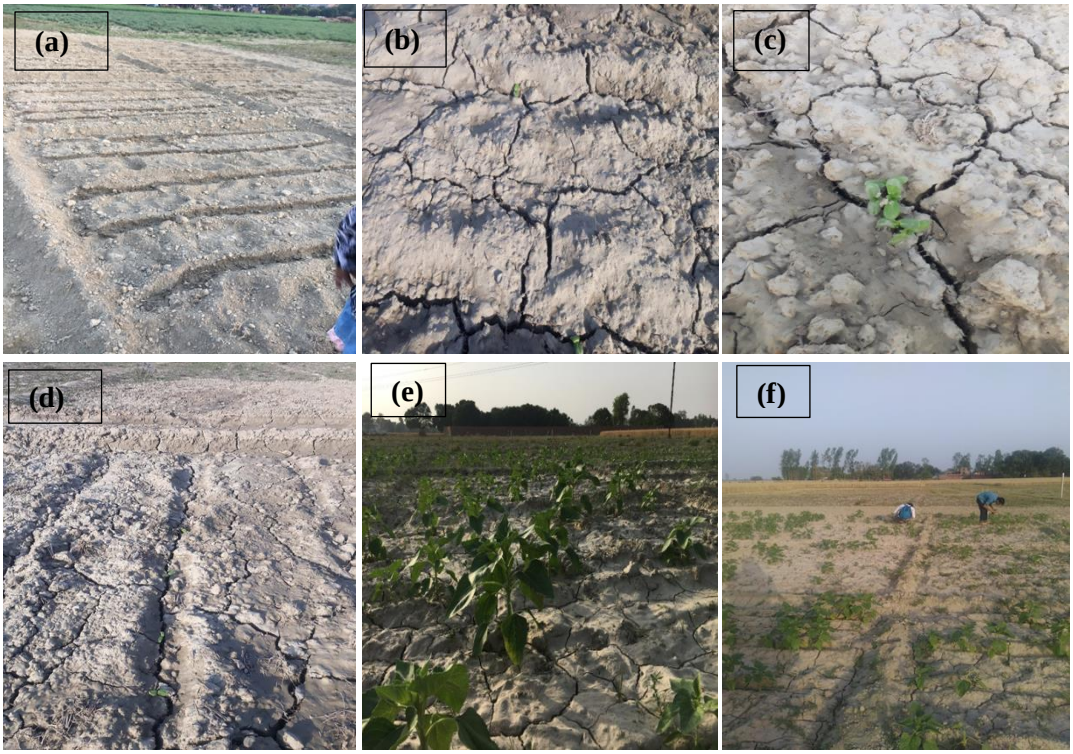
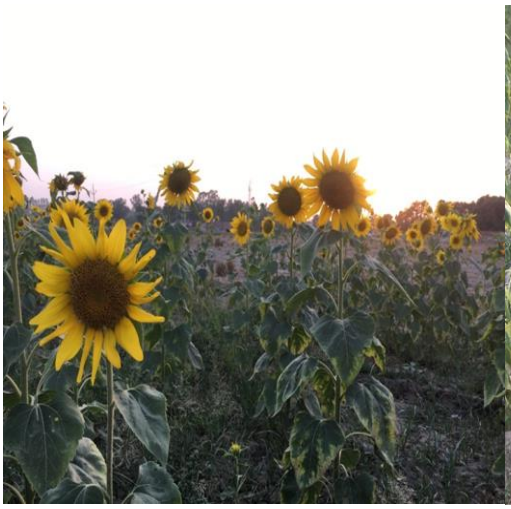


Fig. 37 Experimental field Ambedkar Nagar, Uttar Pradesh, India **(a)** preparation of blocks **(b)** barren condition of the soil **(c)** formation of salt-crusts in soil and germinated sunflower seeds **(d)** white salt crusts in the field **(e)** and **(f)** field after one month of sowing

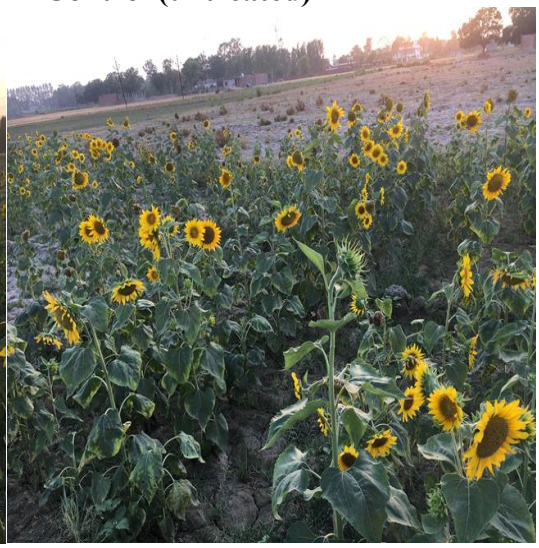
PE3-EPS (at concentration 2%) **Only PE3 treated plants**



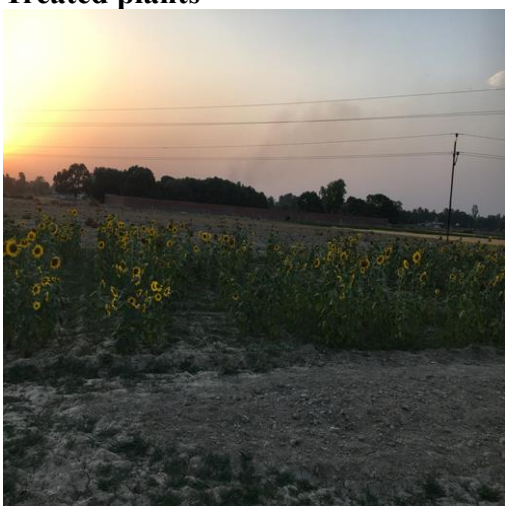
2% EPS + PE3

Control (untreated)

Treated plants



Treated plants

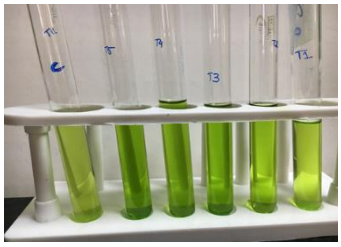


Untreated control (head size)

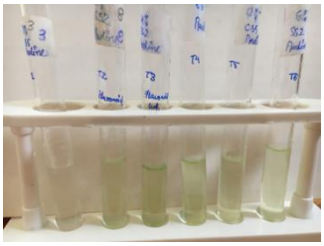
EPS + PE3 (head size)

EPS + PE3 (head size)

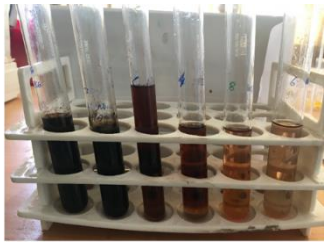
Fig. 38 Effect of various concentrations of EPS, isolates PE3 and AF7 (alone) and in combination with their respective EPS (2% EPS + bacteria), on growth of sunflower plants under saline conditions (field study)



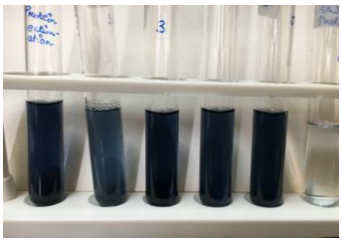
Chlorophyll content



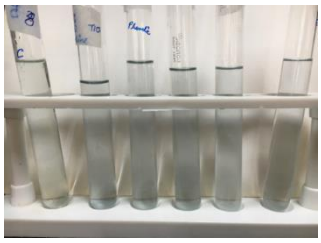
Flavonoid content



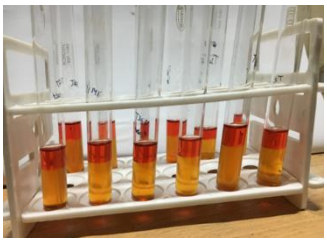
Carbohydrate content



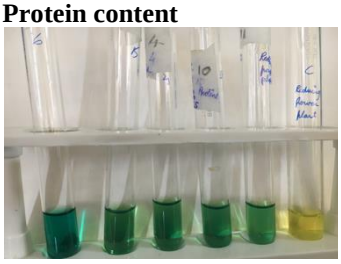
Protein content



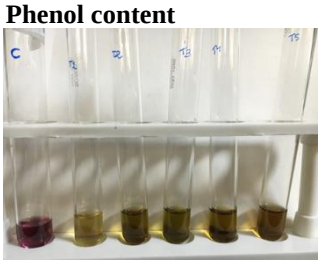
Phenol content



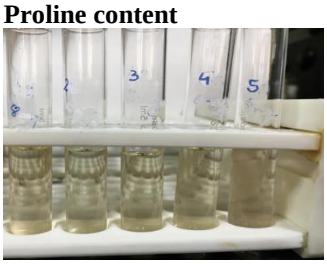
Proline content



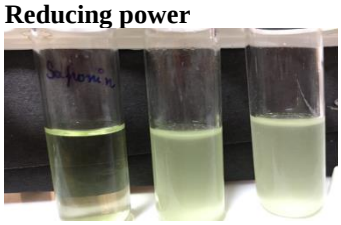
Reducing power



Antioxidant activity



Hydroxyl scavenging activity



Saponins

Steroids and phytosterols

Fig. 39 Effect of various concentrations of EPS, isolates PE3 and AF7 (alone) and in combination with their respective EPS (2% EPS + bacteria), on biochemical and salt-tolerance properties of sunflower plants under saline conditions (field study)

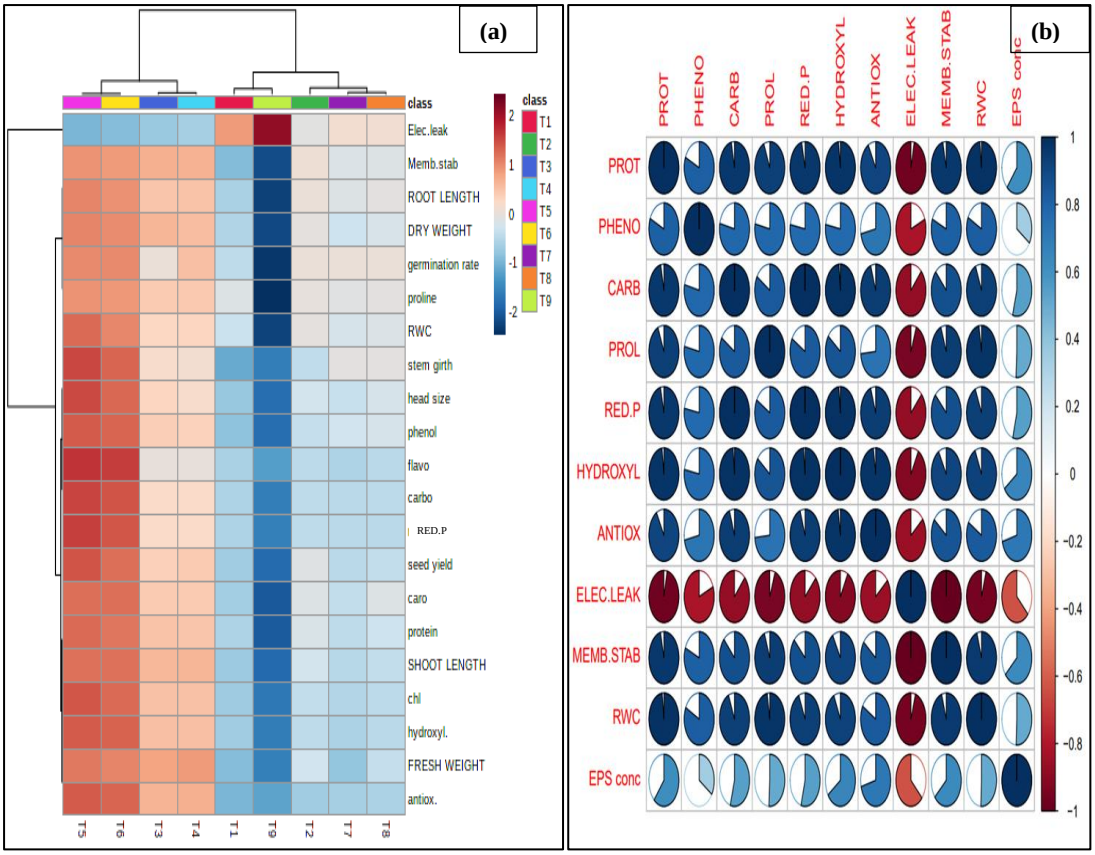


Fig. 40 Determining effect of various EPS concentrations, bacteria (PE3 and AF7) and the combination treatment (2% EPS plus bacteria) on various growth, biochemical and salt-tolerance traits of sunflower plants (**pot study**) under salinity stress **through (a) Heat map based on Pearson and Ward** for determining distance and clustering, (**b) Correlogram** determining the effect of EPS on salt-tolerance properties of inoculated plants

The designated treatments are: T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control. Various plant parameters checked are designated as: chl- chlorophyll content, caro- carotenoid, flavo- flavonoid content, phenol- phenolic content, protein- protein content, carbo- carbohydrate content, proline- proline content, RED.P- reducing power, ANTIOX- antioxidant activity, HYDROXYL- hydroxyl scavenging activity, RWC- relative water content, MEMB.STAB- Membrane stability, EPS conc.- EPS concentrations

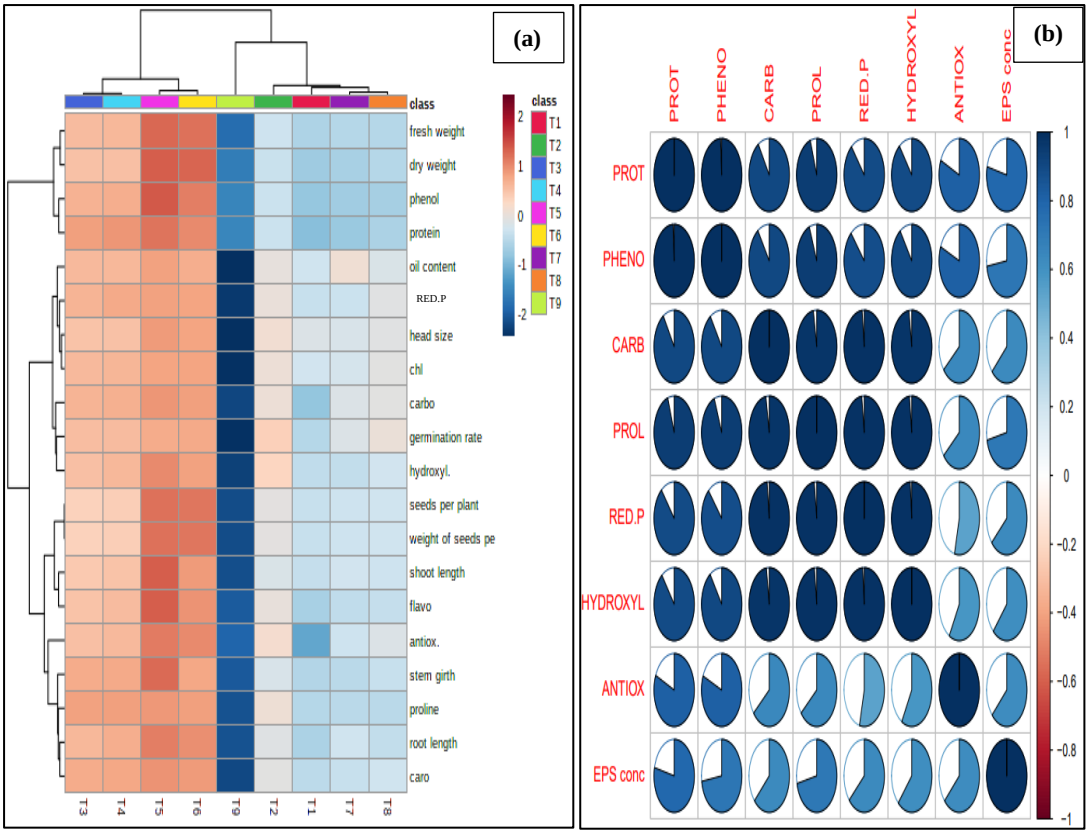


Fig. 41 Determining effect of various EPS concentrations, bacteria (PE3 and AF7) and the combination treatment (2% EPS plus bacteria) on various growth, biochemical and salt-tolerance traits of sunflower plants (**field study**) under salinity stress **through (a) Heat map based on Pearson and Ward** for determining distance and clustering, (**b) Correlogram** determining the effect of EPS on salt-tolerance properties of inoculated plants

The designated treatments are: T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control. Various plant parameters checked are designated as: chl- chlorophyll content, caro- carotenoid, flavo- flavonoid content, phenol- phenolic content, protein- protein content, carbo- carbohydrate content, proline- proline content, RED.P- reducing power, ANTIOX- antioxidant activity, HYDROXYL- hydroxyl scavenging activity, RWC- relative water content, MEMB.STAB- Membrane stability, EPS conc.- EPS concentrations

Table. 2 Soil analysis of sampling sites

Soil Sample id	pH	EC (dS/ m)	Org. Carbon (gm/kg)	Available N (gm/kg)	Available P (mg/kg)	Available K (mg/kg)	Soil organic matter (g/ kg)	Microbial biomass (mg/ kg)
K1	**9.2 ± 0.50**	**10.5 ± 0.76**	3.40 ± 0.05	0.20 ± 0.06	43.0 ± 1.2	152 ± 2.3	5.85 ± 0.03	115.22 ± 1.6
K2	**11.29 ± 0.26**	**16.5 ± 0.54**	3.31 ± 0.13	0.18 ± 0.008	41.2 ± 1.6	155 ± 2.5	5.58 ± 0.05	111.03 ± 2.8
K3	**11.86 ± 0.36**	**19.5 ± 0.39**	3.06 ± 0.08	0.18 ± 0.07	41.1 ± 2.1	155 ± 1.9	5.26 ± 0.07	102.47 ± 2.4
K4	**10.44 ± 1.20**	**12.3 ± 1.20**	3.21 ± 0.09	0.18 ± 0.04	42.6 ± 2.9	152 ± 1.7	5.52 ± 0.08	107.85 ± 3.1
K5	**11.13 ± 0.22**	**12.3 ± 1.30**	3.30 ± 0.15	0.15 ± 0.003	41.3 ± 1.9	156 ± 1.6	5.61 ± 0.50	110.29 ± 4.1
K6	**9.86 ± 0.86**	**11.4 ± 0.91**	3.26 ± 0.23	0.21 ± 0.06	42.9 ± 1.5	152 ± 1.8	5.61 ± 0.30	111.05 ± 2.8

Results are means of three replicates

Data written as mean ± standard deviation (n= 3)

Table. 3 Phenotypic characterization of isolates and their salt-tolerance level

Isolates	Generation time (hours)	Motility	Colony morphology		Microscopic morphology		Growth at 8% NaCl condition	Fluorescence under UV light
			Color	Appearance	Gram nature	Shape		
AF2	4.5	+	Pigmented (Rainbow-colored)	Mucilaginous	Negative	Rod	+	+
AF6	4.1	+	Pigmented (Rainbow-colored)	Mucilaginous	Negative	Rod	+	+
AF7	4.0	+	Pigmented (Rainbow-colored)	Mucilaginous	Negative	Rod	+	+
PE3	4.5	+	Pigmented (slightly yellow)	Mucilaginous	Negative	Rod	+	+
CS4	3.6	+	Pigmented (Yellow)	Mucilaginous	Negative	Rod	-	+
CS5	2.5	+	Pigmented (Yellow)	Mucilaginous	Positive	Rod	+	+
SS2	2.6	+	Pigmented (Yellow)	Mucilaginous	Positive	Rod	+	+
SS6	3.1	+	Non-pigmented (White)	Mucilaginous	Negative	Rod	-	+
S1C	6.1	+	Pigmented (Light yellow)	Mucilaginous	Positive	Rod	+	+
T1K1	4.22	+	Pigmented (Dark-brown)	Non-mucilaginous	Negative	Rod	+	-
T1K2	3.96	-	Non-pigmented (white)	Non-mucilaginous	Negative	Rod	-	-
T1K3	4.88	+	Non-pigmented (white)	Non-mucilaginous	Negative	Rod	-	-
T1K6	2.48	+	Pigmented (Brown)	Mucilaginous	Negative	Rod	-	+
T1K7	3.72	+	Non-pigmented	Mucilaginous	Negative	Rod	-	-

			(white)					
T1K8	4.16	+	Non-pigmented (white)	Mucilaginous	Negative	Rod	-	-
P1A	2.63	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	+	+
P1B	3.54	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	+	+
3T	5.13	+	Non-pigmented (white)	Non-mucilaginous	Negative	Rod	+	+
KS1	2.87	-	Non-pigmented (white)	Non-mucilaginous	Negative	Rod	+	+
KS2	4.24	+	Pigmented (brown)	Mucilaginous	Negative	Rod	-	+
KS3	6.28	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
KS6	4.92	-	Pigmented (yellow)	Mucilaginous	Positive	Rod	-	+
ST6	3.45	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
ST8	2.66	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
Y1M1	2.46	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
Y1M2	3.80	-	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
BER1	4.73	+	Pigmented (brown)	Mucilaginous	Negative	Rod	-	+
VK2	5.28	+	Pigmented (green)	Mucilaginous	Negative	Rod	-	+
MS5	6.04	-	Non-pigmented (white)	Mucilaginous	Negative	Rod	-	+

KD1	4.11	+	Non-pigmented (white)	Mucilaginous	Negative	Rod	-	+
KD2	2.04	-	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
KD8	2.61	-	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
K1A	3.85	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	-
K1B	4.79	+	Pigmented (green)	Mucilaginous	Negative	Rod	-	-
K1C	4.23	+	Non-pigmented (white)	Mucilaginous	Negative	Rod	-	+
K1F	4.44	-	Pigmented (green)	Mucilaginous	Negative	Rod	-	+
K2B	3.84	+	Non-pigmented (white)	Mucilaginous	Negative	Rod	-	+
K3D	2.14	+	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	-
B1S2	2.63	-	Pigmented (yellow)	Mucilaginous	Negative	Rod	-	+
B1S5	2.79	-	Pigmented (green)	Mucilaginous	Negative	Rod	-	+
B1S6	4.08	+	Pigmented (green)	Mucilaginous	Negative	Rod	-	+
B1S8	4.68	+	Pigmented (green)	Mucilaginous	Negative	Rod	-	+

+ indicates positive for test
- indicates negative for test

Table. 4 Growth pattern of isolates at various pH and temperature

Isolates	pH variants					Temperature (° C)				
	4	**6**	**8**	**10**	**12**	**5**	**15**	**25**	**35**	**45**
AF2	-	+	+	+	-	-	+	+	+	-
AF6	-	+	+	+	+	-	+	+	+	+
AF7	-	+	+	+	-	-	+	+	+	-
PE3	-	+	+	+	-	-	+	+	+	-
CS4	-	+	+	+	-	-	+	+	+	-
CS5	+	+	+	+	+	-	+	+	+	-
SS2	-	+	+	+	-	-	+	+	+	-
SS6	-	+	+	+	-	-	+	+	+	-
S1C	-	+	+	+	+	-	+	+	+	-
T1K1	+	+	+	+	-	-	+	+	+	-
T1K2	-	+	+	+	-	-	+	+	+	+
T1K3	-	+	+	+	-	+	+	+	+	-
T1K6	-	+	+	+	-	-	+	+	+	-
T1K7	-	+	+	+	-	-	+	+	+	-
T1K8	-	+	+	+	+	-	+	+	+	-
P1A	-	+	+	+	-	-	+	+	+	-
P1B	-	+	+	+	-	-	+	+	+	-
3T	+	+	+	+	-	-	+	+	+	+
KS1	-	+	+	+	+	+	+	+	+	-
KS2	-	+	+	+	-	-	+	+	+	-
KS3	-	+	+	+	-	-	+	+	+	-
KS6	+	+	+	+	+	-	+	+	+	-
ST6	-	+	+	+	-	-	+	+	+	-
ST8	-	+	+	+	-	-	+	+	+	-
Y1M1	-	+	+	+	-	-	+	+	+	-

Y1M2	+	+	+	+	+	-	+	+	+	+
BER1	-	+	+	+	-	-	+	+	+	-
VK2	-	+	+	+	-	-	+	+	+	-
MS5	-	+	+	+	+	-	+	+	+	-
KD1	-	+	+	+	-	-	+	+	+	-
KD2	-	+	+	+	-	-	+	+	+	-
KD8	-	+	+	+	+	-	+	+	+	-
K1A	-	+	+	+	+	-	+	+	+	-
K1B	-	+	+	+	-	-	+	+	+	-
K1C	+	+	+	+	-	-	+	+	+	-
K1F	-	+	+	+	-	-	+	+	+	-
K2B	-	+	+	+	+	-	+	+	+	-
K3D	-	+	+	+	-	-	+	+	+	+
B1S2	-	+	+	+	-	-	+	+	+	-
B1S5	-	+	+	+	-	-	+	+	+	-
B1S6	-	+	+	+	-	-	+	+	+	-
B1S8	-	+	+	+	-	-	+	+	+	-

+ indicates utilized

- indicates not utilized

Table. 5 Utilization of various carbon sources by isolates

	Carbon sources												
Isolates	**Mannitol**	**Glucose**	**Dextrose**	**Lactose**	**Sucrose**	**Galactose**	**Maltose**	**Starch**	**Citrate**	**Malate**	**Trehalose**	**Fructose**	**Glycerol**
AF2	+	-	-	-	+	-	-	-	+	-	+	-	+
AF6	+	+	-	-	+	+	-	-	+	-	+	+	+
AF7	+	-	-	-	+	-	-	-	+	-	+	-	+
PE3	+	+	-	+	+	+	-	-	+	+	-	+	+
CS4	-	+	-	+	-	-	+	-	+	-	-	-	-
CS5	+	+	+	-	+	+	-	+	-	-	+	+	+
SS2	+	+	+	-	+	+	-	+	-	-	+	+	+
SS6	-	+	-	-	-	+	-	+	+	-	+	-	+
S1C	+	+	-	+	+	+	+	-	+	-	-	+	+
T1K1	+	+	+	-	-	+	-	-	-	-	+	+	+
T1K2	+	+	-	+	+	+	+	-	+	-	-	+	+
T1K3	+	+	+	-	+	+	-	+	-	-	+	+	+
T1K6	+	-	-	-	+	-	-	-	+	-	+	-	-
T1K7	+	+	+	-	+	+	-	+	-	-	+	+	+
T1K8	+	+	+	-	+	+	-	+	-	-	+	+	+
P1A	+	+	+	-	+	+	-	+	-	-	+	-	+
P1B	+	+	-	+	+	+	+	-	+	-	-	+	-
3T	+	+	+	-	+	+	-	+	-	-	+	+	+
KS1	+	+	-	+	+	+	+	-	+	-	-	-	+
KS2	+	-	-	-	+	-	-	-	+	-	+	-	-
KS3	+	+	+	-	+	+	-	+	-	-	+	+	+

KS6	-	+	-	+	-	-	+	-	+	-	-	-	-
ST6	+	-	-	-	+	-	-	-	+	-	+	-	+
ST8	-	+	-	+	-	-	+	-	+	-	-	-	-
Y1M1	+	+	+	-	+	+	-	+	-	-	+	+	+
Y1M2	+	-	-	-	+	-	-	-	+	-	+	-	+
BER1	+	+	+	+	+	+	+	-	-	-	-	+	+
VK2	-	+	-	+	-	-	+	-	+	-	-	-	-
MS5	-	+	-	+	-	-	+	-	+	-	-	-	-
KD1	+	+	+	-	+	+	-	-	-	-	+	+	-
KD2	+	-	-	-	+	-	-	-	+	-	+	-	+
KD8	-	+	-	-	-	+	-	+	+	-	+	+	+
K1A	+	+	-	+	+	+	+	-	+	-	-	+	-
K1B	-	+	+	-	-	+	-	+	-	-	+	+	+
K1C	+	+	-	-	+	+	-	-	+	-	+	+	+
K1F	-	+	-	+	-	-	+	-	-	-	-	-	-
K2B	+	+	-	+	+	+	+	-	+	-	-	+	+
K3D	-	+	-	-	-	+	-	+	+	-	+	+	+
B1S2	+	+	-	-	+	+	-	-	-	-	+	+	-
B1S5	+	+	-	+	+	+	+	-	+	-	-	+	+
B1S6	+	+	+	+	+	+	+	-	-	-	-	+	+
B1S8	-	+	-	+	-	-	+	-	+	-	-	-	-

+ =utilized, - = not utilized

Table. 6 Utilization of various nitrogen sources by isolates

	Nitrogen sources										
Isolates	**Yeast extract**	**Potassium nitrate (KNO_3)**	**Sodium nitrate ($NaNO_3$)**	**Tryptophan**	**Ammonium chloride (NH_4Cl)**	**Glycine**	**Ammonium sulphate ($(NH_4)_2SO_4$**	**Lysine**	**Glutamine**	**Cysteine**	**Methionine**
AF2	+	+	+	+	+	-	+	+	+	+	-
AF6	+	+	+	+	+	-	+	+	+	+	-
AF7	+	+	+	+	+	-	+	+	+	+	-
PE3	+	+	+	+	+	-	+	+	+	+	-
CS4	+	+	+	+	+	-	+	+	+	-	-
CS5	+	+	+	+	+	-	+	+	+	-	-
SS2	+	+	+	+	+	-	+	+	+	-	-
SS6	+	+	+	+	+	-	+	+	+	-	-
S1C	+	+	+	+	+	-	+	+	-	-	-
T1K1	+	+	+	+	+	-	+	+	+	-	+
T1K2	+	+	+	+	+	-	+	+	+	-	-
T1K3	+	+	+	+	+	-	+	+	-	+	-
T1K6	+	-	-	+	+	-	+	+	-	+	+
T1K7	+	+	+	+	+	+	+	+	-	-	+
T1K8	+	+	-	+	+	-	+	+	-	-	-
P1A	+	+	+	+	+	-	+	+	-	+	+
P1B	+	+	+	+	+	+	+	+	-	-	-
3T	+	+	+	+	+	-	+	+	-	+	-
KS1	+	+	+	+	+	-	+	+	-	-	-
KS2	+	+	+	+	+	-	-	-	+	-	-
KS3	+	+	+	+	+	+	+	+	-	-	-
KS6	+	+	-	+	+	-	+	-	+	-	-
ST6	+	+	+	+	+	-	-	+	-	-	+
ST8	+	+	-	+	+	-	+	-	+	+	-
Y1M1	+	+	+	+	+	-	+	+	-	-	-
Y1M2	+	+	+	+	+	+	+	-	-	-	-
BER1	+	+	+	+	+	-	+	+	-	-	-

VK2	+	+	+	+	+	-	+	-	+	+	-
MS5	+	+	+	+	+	-	+	-	-	-	-
KD1	+	+	+	+	+	-	+	+	-	-	+
KD2	+	+	-	+	+	-	+	+	-	-	-
KD8	+	+	+	+	+	+	+	-	+	+	-
K1A	+	+	+	+	+	-	+	+	-	-	-
K1B	+	+	+	+	+	-	+	+	-	-	+
K1C	+	+	+	+	+	-	-	+	-	+	-
K1F	+	+	+	+	+	-	+	-	-	-	-
K2B	+	+	+	+	+	-	+	-	+	-	-
K3D	+	+	+	+	+	+	+	+	-	-	-
B1S2	+	+	+	+	+	-	-	-	-	+	-
B1S5	+	+	-	+	+	-	+	+	-	-	-
B1S6	+	+	+	+	+	-	+	+	-	-	-
B1S8	+	+	+	+	+	-	+	+	+	+	-

+ =utilized, - = not utilized

Table. 7 Biochemical characterization of the isolates

Isolates	Catalase	Oxidase	Lactose fermentation	Growth on GPA	Nitrate reductase	Urease	Gelatinase	Citrate	Amylase	Lipase	Protease	Cellulase	Ammonia production	Indole test	H_2S production	Methyl red	Voges Proskauer	PHB accumulation
AF2	+	+	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	+
AF6	+	+	-	+	+	-	-	+	+	+	-	-	+	-	+	-	-	+
AF7	+	+	-	+	-	-	-	+	+	+	-	-	+	+	-	-	+	+
PE3	+	+	+	+	+	-	-	+	-	+	-	+	+	+	-	-	-	+
CS4	+	-	-	+	+	-	-	+	+	+	-	+	+	-	-	-	+	+
CS5	+	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+
SS2	+	-	-	-	+	-	+	-	+	-	-	-	+	+	-	-	+	+
SS6	+	+	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	+
S1C	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	-	+	+
T1K1	+	+	+	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+
T1K2	+	+	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+	+
T1K3	+	+	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+
T1K6	+	-	-	+	-	-	-	+	+	-	+	-	+	+	-	-	+	+
T1K7	+	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-	+	+
T1K8	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	+	+
P1A	+	+	-	-	-	-	+	+	+	-	+	-	+	+	+	-	+	-
P1B	-	+	+	-	+	+	-	+	+	-	-	-	+	-	-	-	+	-
3T	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+	+
KS1	-	-	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	+
KS2	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	+	+
KS3	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	-	+	+
KS6	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	+	+
ST6	+	-	-	+	+	-	-	+	+	-	+	-	+	+	-	-	+	-

ST8	+	-	-	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+
Y1M1	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	+	+	+
Y1M2	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	+
BER1	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-	-	+	+
VK2	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	+	+
MS5	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-
KD1	+	-	+	+	-	-	-	+	+	-	+	-	+	-	-	-	+	-
KD2	+	-	-	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
KD8	+	-	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	+
K1A	+	-	-	+	-	-	-	+	+	-	-	+	+	-	-	-	-	+
K1B	+	+	-	+	-	-	-	+	+	-	+	-	+	+	-	-	+	+
K1C	+	-	-	-	+	-	-	+	+	+	-	-	+	-	-	+	-	+
K1F	+	+	-	+	-	-	-	-	+	-	-	+	+	-	-	-	+	+
K2B	+	-	-	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-
K3D	+	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	+	+
B1S2	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+
B1S5	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	-	+
B1S6	+	+	-	+	+	-	-	+	+	-	-	-	+	+	-	-	+	-
B1S8	+	-	-	-	-	-	+	+	+	+	-	-	+	+	+	-	+	+

+ indicates positive for test - indicates negative for test

Table. 8 Plant growth promoting characteristics of isolates

Isolates	Phosphate solubilization (µg phosphate solubilized)	Zinc solubilization (zsi)	Siderophore production (psu)	Potassium solubilization (ksi)	IAA production (µg/ml)	Gibberellic acid production (µg/ml)	EPS production (g/100ml)	HCN production
AF2	56.11 ± 0.12	1.26 ± 0.06	23.62 ± 0.02	-	10.34 ± 0.01	88.46 ± 0.85	0.08 ± 0.006	-
AF6	-	1.18 ± 0.12	42.07 ± 0.66	-	12.25 ± 0.13	91.26 ± 1.2	0.007 ± 0.0001	-
AF7	**126.03 ± 0.1**	**2.79 ± 0.2**	**95.28 ± 1.45**	**-**	**29.04 ± 0.04**	**160.55 ± 2.1**	**0.916 ± 0.09**	**+**
PE3	**309.12 ± 3.82**	**4.40 ± 0.09**	**72.45 ± 1.49**	**6.44 ± 0.67**	**130.87 ± 0.20**	**165.53 ± 1.03**	**0.54 ± 0.07**	**+**
CS4	43.67 ± 0.86	-	36.19 ± 0.05	-	-	58.10 ± 1.2	0.045 ± 0.008	+
CS5	101.58 ± 0.29	2.2 ± 0.14	58.21 ± 2.10	-	23.11 ± 0.04	94.27 ± 0.82	0.14 ± 0.08	+
SS2	115.22 ± 0.14	3.1 ± 0.22	44.93 ± 1.09	-	20.84 ± 0.27	121.0 ± 1.57	0.28 ± 0.04	+
SS6	72.06 ± 0.88	1.05 ± 0.03	20.63 ± 0.06	-	-	46.12 ± 0.25	0.003 ± 0.0006	+
S1C	125.41 ± 0.11	2.11 ± 0.15	92.32 ± 0.12	5.91 ± 0.03	20.81 ± 0.04	95.46 ± 0.17	0.23 ± 0.005	+
T1K1	119.48 ± 1.2	2.79 ± 0.20	89.05 ± 0.13	6.06 ± 0.15	45.48 ± 0.12	102.55 ± 0.26	0.35 ± 0.012	+
T1K2	-	-	-	20.26 ± 0.05	86.07 ± 0.61	-	0.003 ± 0.0001	+
T1K3	48.05 ± 0.54	2.05 ± 0.13	26.92 ± 0.50	-	30.53 ± 0.08	-	0.12 ± 0.006	+
T1K6	52.86 ± 0.27	2.07 ± 0.22	38.10 ± 0.12	-	11.08± 0.14	26.45 ± 0.22	0.21 ± 0.004	+
T1K7	-	1.16 ± 0.06	45.19 ± 0.09	-	9.14 ± 0.002	68.57 ± 1.2	0.005 ± 0.0002	+
T1K8	33.91 ± 0.38	1.54 ± 0.16	56.32 ± 0.14	-	-	53.85 ± 0.14	0.05 ± 0.003	+
P1A	120.46 ± 0.42	1.49 ± 0.22	32.61 ± 0.13	-	14.82 ± 0.13	34.79 ± 0.18	0.36 ± 0.03	+
P1B	105.36 ± 0.18	1.08 ± 0.08	22.96 ± 0.20	-	20.63 ± 0.004	56.07 ± 0.003	0.26 ± 0.06	+
3T	98.27 ± 0.74	2.11 ± 0.17	12.41 ± 0.06	-	16.71 ± 0.008	86.05 ± 0.09	0.46 ± 0.08	+
KS1	82.41 ± 0.43	1.37 ± 0.23	32.48 ± 0.12	-	22.46 ± 0.002	43.16 ± 0.09	0.42 ± 0.13	+
KS2	42.73 ± 0.05	1.07 ± 0.18	36.96 ± 0.22	-	-	-	0.12 ± 0.002	+
KS3	-	1.86 ± 0.19	68.34 ± 0.06	-	10.56 ± 0.12	-	0.06 ± 0.001	+
KS6	36.92 ± 0.11	1.93 ± 0.08	-	-	18.73 ± 0.08	-	0.16 ± 0.009	-
ST6	53.14 ± 0.29	2.10 ± 0.07	34.82 ± 0.47	-	-	-	0.07 ± 0.005	+
ST8	-	-	76.28 ± 0.12	-	-	-	0.14 ± 0.007	-

Y1M1	86.19 ± 0.34	-	83.04 ± 0.17	10.36 ± 0.15	11.48 ± 0.23	-	0.03 ± 0.006	-
Y1M2	25.67 ± 0.40	-	96.02 ± 0.05	-	45.88 ± 0.22	53.12 ± 0.12	0.19 ± 0.002	-
BER1	-	1.48 ± 0.23	-	-	-	40.32 ± 0.006	0.04 ± 0.003	-
VK2	-	1.96 ± 0.19	23.18 ± 0.8	-	82.44 ± 0.15	63.09 ± 0.04	0.22 ± 0.01	+
MS5	73.96 ± 0.48	1.58 ± 0.48	16.17 ± 0.12	-	101.39 ± 0.14	32.96 ± 0.01	0.06 ± 0.005	+
KD1	105.12 ± 1.1	1.16 ± 0.06	66.25 ± 0.06	-	10.22 ± 0.008	24.09 ± 0.002	0.22 ± 0.012	+
KD2	-	1.23 ± 0.23	72.94 ± 0.12	-	5.11 ± 0.007	36.76 ± 0.003	0.09 ± 0.033	+
KD8	82.04 ± 0.68	1.42 ± 0.13	88.14 ± 0.06	-	-	15.68 ± 0.02	0.072 ± 0.001	+
K1A	71.06 ± 0.92	-	90.11 ± 0.17	-	-	39.19 ± 0.09	0.16 ± 0.004	-
K1B	-	1.92 ± 0.33	28.47 ± 0.04	-	-	-	0.082 ± 0.006	+
K1C	62.64 ± 0.19	1.86 ± 0.08	-	8.22 ± 0.08	-	78.46 ± 0.15	0.096 ± 0.004	+
K1F	-	1.99 ± 0.12	11.28 ± 0.05	-	35.64 ± 0.012	-	0.24 ± 0.05	-
K2B	58.19 ± 0.73	2.16 ± 0.23	35.84 ± 0.04	-	16.55 ± 0.06	82.19 ± 0.86	0.16 ± 0.006	-
K3D	-	-	-	-	29.26 ± 0.04	43.97 ± 0.06	0.096 ± 0.007	-
B1S2	-	1.26 ± 0.08	24.79 ± 0.06	-	23.48 ± 0.11	-	0.084 ± 0.003	+
B1S5	99.12 ± 0.53	1.29 ± 0.06	-	-	-	55.47 ± 0.36	0.173 ± 0.005	-
B1S6	46.81 ± 0.61	1.94 ± 0.20	-	-	-	62.41 ± 0.09	0.063 ± 0.007	+
B1S8	62.36 ± 0.70	2.06 ± 0.12	86.73 ± 0.12	-	36.78 ± 0.06	71.96 ± 0.006	0.11 ± 0.003	-

***+** indicates positive for test, **-** indicates negative for test, zsi= zinc solubilization index, psu= percent siderophore unit, ksi= potassium solubilization index,*

Values written as mean ± standard deviation (n= 3)

Table. 9 Effect of salinity on various plant growth promoting properties of selected isolates (PE3 and AF7) and the estimation of most dominant responses of the isolates to salinity stress

Isolate PE3					
Properties	**Non-saline control**	**2% NaCl**	**4% NaCl**	**6% NaCl**	**8% NaCl**
Phosphate solubilization (µg phosphate solubilized)	**309.12 ± 3.82**	**406.64 ± 8.11**	**201.91 ± 7.16**	**72.38 ±7.65**	**-**
Zinc solubilization index (zsi)	4.40 ± 0.09	5.64 ± 0.17	4.69 ± 0.51	3.08 ± 0.27	-
Potassium solubilization index (ksi)	6.44 ± 0.67	7.33 ± 0.27	5.14 ± 0.23	-	-
GA production (µg/ml)	165.53 ± 1.03	182.49 ± 5.55	158.77 ± 3.20	133.08 ± 5.46	98.49 ± 4.89
IAA production (µg/ml)	130.87 ± 0.20	161.35 ± 0.55	128.04 ± 2.97	111.02 ± 1.54	77.68 ± 2.25
EPS production (g/100ml)	**0.54 ± 0.07**	**1.25 ± 0.05**	**0.55 ± 0.08**	**0.21 ± 0.03**	**0.06 ± 0.02**
Siderophore production (percent siderophore unit)	72.45 ± 1.49	74.81 ± 1.54	69.24 ± 1.54	64.74 ± 4.62	-
HCN	+++	++	+	+	+

Isolate AF7					
Properties	**Non-saline control**	**2% NaCl**	**4% NaCl**	**6% NaCl**	**8% NaCl**
Phosphate solubilization (µg phosphate solubilized)	126.03 ± 0.1	150.58 ± 0.2	-	-	-
Zinc solubilization index	2.79 ± 0.2	3.26 ± 0.2	2.8 ± 0.10	2.4 ± 0.1	-
Potassium solubilization	Absent	Absent	Absent	Absent	Absent
GA production (µg/ml)	**160.55 ± 2.1**	**349.78 ± 2.94**	**310.09 ± 1.33**	**218.05 ± 3.6**	**144.14 ± 2.08**
IAA production (µg/ml)	29.04 ± 0.04	36.41 ± 0.5	33.92 ± 0.20	24.22 ± 0.46	17.06 ± 0.65
EPS production (g/100ml)	**0.916 ± 0.09**	**3.32 ± 0.12**	**2.76 ± 0.11**	**1.11 ± 0.09**	**0.5±0.12**
Siderophore production (percent siderophore unit)	95.28 ± 1.45	97.29 ± 0.50	56.06 ± 3.43	11.15 ± 12.26	-
HCN production	+++	++	-	-	-

Results expressed as mean ± S.D (n=3)

+++= high, ++= medium, += low, -= absent

The parameters in bold are reported as most primary and dominant responses of isolates against increasing saline conditions

Table. 10 Effect of salinity on various salt-tolerance properties of selected isolates (PE3 and AF7) and the estimation of most dominant responses of the isolates to salinity stress

Isolate PE3					
Salt-tolerance properties	**Non-saline control**	**2% NaCl**	**4% NaCl**	**6% NaCl**	**8% NaCl**
Sodium accumulation (µg/gm of cell dry weight)	**3522.24**	**8205.87**	**12521.06**	**32591.72**	**23434.93**
Proline (µg/mg protein)	**103.71 ± 0.82**	**191.38 ± 0.77**	**239.56 ± 0.31**	**365.91 ± 5.05**	**301.39 ± 4.81**
Antioxidant activity (% inhibition)	66.92 ± 1.35	71.39 ± 1.00	82.38 ± 1.40	80.61 ± 0.82	87.96 ± 0.95
Hydroxyl scavenging activity (%)	83.03 ± 0.69	85.04 ± 0.21	94.23 ± 0.57	97.55 ± 0.12	97.90 ± 0.21
Reducing power (quercetin equivalent mg/ml)	3.3 ± 0.07	3.45 ± 0.13	2.90 ± 0.06	2.29 ± 0.02	1.95 ± 0.01
Biofilming ability (Optical density)	2.77 ± 0.073	2.95 ± 0.117	3.16 ± 0.033	0.62 ± 0.002	0.61 ± 0.007
Isolate AF7					
Salt-tolerance properties	**Non-saline control**	**2% NaCl**	**4% NaCl**	**6% NaCl**	**8% NaCl**
Sodium accumulation (µg/gm of cell dry weight)	**4292.21**	**11124.58**	**13857.26**	**34024.57**	**26465.53**
Proline (µg/mg protein)	125.80 ± 0.59	221.31 ± 1.09	234.40 ± 6.21	298.37 ± 2.91	300.25 ± 8.34
Antioxidant activity (% inhibition)	65.22 ± 3.90	70.46 ± 0.87	98.42 ± 1.79	101.26 ± 0.48	110.99 ± 1.00
Hydroxyl scavenging activity (% inhibition)	81.065 ± 0.80	83.74 ± 0.25	84.57 ± 0.06	93.46 ± 0.52	96.06 ± 0.24
Reducing power (quercetin equivalent mg/ml)	3.69 ± 0.29	4.03 ± 0.22	3.85 ± 0.28	3.45 ± 0.09	2.57 ± 0.01
Biofilming ability (Optical density)	**0.821 ± 0.004**	**2.88 ± 0.017**	**3.16 ± 0.089**	**1.01 ± 0.001**	**1.08 ± 0.004**

Results expressed as mean ± S.D (n=3)

The parameters in bold are reported as most primary and dominant responses of isolates against increasing saline conditions

Table. 11 Biocontrol activity of isolates against common phytopathogens

	Phytopathogens (% radial growth inhibition)			
Isolate	***Macrophomina phaseolina***	***Fusarium solani***	***Fusarium oxysporum***	***Alternaria brassicae***
AF2	+ (38%)	+ (23%)	+ (49%)	-
AF6	-	+ (33%)	+ (52%)	+ (44%)
AF7	+ (48%)	-	+ (51%)	-
PE3	+ (55%)	-	+ (60%)	+ (63%)
CS4	+ (48%)	+ (56%)	-	+ (60%)
CS5	+ (59%)	+ (63%)	-	+ (24%)
SS2	+ (44%)	+ (54%)	+ (41%)	-
SS6	+ (23%)	+ (40%)	-	+ (33%)
S1C	+ (56%)	+ (35%)	-	-
T1K1	+ (63%)	+ (78%)	+ (59%)	+ (53%)
T1K2	+ (48%)	+ (22%)	+ (49%)	+ (32%)
T1K3	-	-	+ (31%)	-
T1K6	+ (25%)	+ (41%)	+ (29%)	-
T1K7	+ (21%)	-	-	-
T1K8	-	+ (54%)	+ (32%)	+ (29%)
P1A	-	-	+ (49%)	-
P1B	+ (39%)	+ (46%)	+ (58%)	+ (37%)
3T	-	+ (60%)	+ (16%)	-
KS1	+ (25%)	+ (45%)	+ (23%)	-
KS2	-	-	+ (48%)	-
KS3	+ (19%)	+ (62%)	+ (48%)	-
KS6	+ (21%)	+ (30%)	+ (52%)	-
ST6	+ (56%)	+ (42%)	+ (55%)	+ (35%)
ST8	+ (63%)	+ (22%)	+ (30%)	-
Y1M1	-	-	+ (14%)	-
Y1M2	+ (41%)	+ (23%)	+ (30%)	-
BER1	-	+ (10%)	-	+ (46%)
VK2	-	-	+ (13%)	-
MS5	+ (11%)	+ (49%)	+ (62%)	+ (65%)
KD1	+ (27%)	+ (58%)	+ (68%)	-
KD2	+ (36%)	-	+ (28%)	-
KD8	+ (58%)	+ (34%)	+ (61%)	-
K1A	+ (30%)	-	-	-
K1B	+ (49%)	+ (20%)	+ (29%)	-
K1C	+ (36%)	+ (72%)	+ (62%)	+ (48%)
K1F	+ (22%)	+ (36%)	+ (45%)	-
K2B	+ (49%)	-	+ (62%)	+ (32%)
K3D	-	+ (42%)	+ (21%)	+ (40%)
B1S2	+ (17%)	-	+ (47%)	+ (56%)
B1S5	+ (28%)	+ (39%)	+ (55%)	+ (62%)
B1S6	+ (28%)	-	+ (22%)	+ (48%)
B1S8	+ (54%)	-	+ (31%)	+ (61%)

+ indicates positive for test

- indicates negative for test

Table. 12 Molecular and proteomic characterization and the NCBI-GenBank and culture collection accession number details of isolates

Isolates id	**Organism**	**Identification method**	**Identification score**	**Accession number (GenBank)**	**Culture collection accession number (NAIMCC, Mau, India)**
PE3	***Pseudomonas entomophila***	**MALDI-TOF MS biotyping/ 16S rRNA sequencing**	**1.946/ 99.92%**	**MN515387**	**NAIMCC-B-02324**
AF7	***Alcaligenes faecalis***	**MALDI-TOF MS biotyping/ 16S rRNA sequencing**	**2.254/ 99%**	**MK898928**	**NAIMCC-B-02325**
AF2	*Alcaligenes faecalis*	16S rRNA sequencing	99%	-	
S1C	*Cellulomonas pakistanensis*	16S rRNA sequencing	92.78%	-	
CS5	*Bacillus pumilus*	MALDI-TOF MS	1.978	-	
SS2	*Bacillus pumilus*	MALDI-TOF MS	1.876	-	

Table. 13 Changes in composition and salt-tolerance properties of PE3 extracted EPS under various saline conditions

Components	0% NaCl EPS	2% NaCl EPS	4% NaCl EPS	6% NaCl EPS	8% NaCl EPS
Carbohydrate content (mg/gm)	6266.67 ± 102.74	**9433.33 ± 154.56**	6650.00 ± 81.65	5216.67 ± 62.36	2316.67 ± 47.14
Protein content (mg/gm)	2973.17 ± 7.66	**4469.67 ± 15.79**	3551.17 ± 22.69	2601 ± 20.20	1790.50 ± 29.09
Phenolic content (mg/gm)	15.73 ± 0.54	**20.03 ± 1.53**	14.98 ± 0.06	13.29 ± 0.02	13.16 ± 0.06
Sodium accumulation (mg /Kg of dry weight eps)	1863 ± 10.53	**37525 ± 25.81**	31787 ± 19.62	28423 ± 24.96	21497 ± 12.47
Reducing power (mg/ gm)	850 ± 12.25	**2241.67 ± 31.18**	1750 ± 40.82	1011.67 ± 27.79	391.67 ± 11.79
Antioxidant activity (% inhibition)	66.67 ± 2.53	**83.72 ± 1.27**	72.09 ± 2.90	70.28 ± 1.59	52.71 ± 2.76
Hydroxyl scavenging activity (% inhibition)	80.49 ± 1.18	**87.75 ± 0.87**	82.76 ± 2.41	77.25 ± 1.87	70.60 ± 1.93
Flocculation (%)	68.44 ± 0.11	**70.18 ± 1.26**	69.04 ± 0.17	66.26 ± 0.25	61.72 ± 0.52
Emulsification activity (%)	53.33 ± 6.00	**57.75 ± 8.89**	53.03 ± 2.14	37.58 ± 7.62	23.74 ± 5.00

Results expressed as mean ± S.D (n=3)
Values in bold indicate the highest stimulation in properties upon exposure to salinity

Table. 14 XRD analysis of PE3-EPS under various salt-conditions and estimation of crystallinity and structure of the polymer

PE3-EPS at various saline conditions	Degree of Crystallinity (DOC) (%)	Amorphous Content (Weight %)	Crystallite size (nm)
Non-salinized EPS	41.65	58.35	18.306
2% NaCl-EPS	45.10	54.90	26.468
4% NaCl-EPS	58.24	35.55	35.554
6% NaCl-EPS	45.84	54.16	24.706
8% NaCl-EPS	46.31	53.69	16.316

Table. 15 Difference in elementary composition of PE3-EPS extracted under various saline conditions through SEM-EDS analysis

PE3-EPS at various saline conditions	Carbon		Oxygen		Sodium		Chlorine	
	Wt%	**At %**	**Wt%**	**At %**	**Wt%**	**At %**	**Wt%**	**At %**
Non-salinized EPS	52.74	64.45	32.66	29.97	6.86	4.38	1.84	0.76
2% NaCl-EPS	52.21	67.25	18.91	18.29	14.71	9.90	9.65	4.21
4% NaCl-EPS	47.59	67.05	9.74	10.31	19.11	14.07	16.74	7.99
6% NaCl-EPS	52.82	65.75	23.38	21.85	13.78	8.96	2.29	3.26
8% NaCl-EPS	66.24	77.60	16.96	14.91	7.31	4.48	7.17	2.84

Wt%- Weight percent
At%- Atomic percent

Table. 16 Effect of EPS (at various concentrations) and selected isolates *Pseudomonas entomophila* PE3 and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on growth parameters and productivity of sunflower under saline conditions (pot study)

Treatments	Root length (cm)	Shoot length (cm)	Fresh weight (gm)	Dry weight (gm)	Stem girth (cm)	Head size (cm)	Germination rate* (%)	Seed yield (g/ pot)
T1	5.37 ± 0.09^{b}	43.50 ± 2.36^{b}	14.99 ± 0.15^{b}	7.12 ± 0.31^{b}	1.60 ± 0.08^{b}	2.50 ± 0.16^{b}	$53.33 \pm 9.43^{a,b}$	5.14 ± 0.10^{b}
T2	6.37 ± 0.12^{c}	52.27 ± 1.17^{d}	16.09 ± 0.28^{c}	7.81 ± 0.03^{c}	1.97 ± 0.12^{c}	2.93 ± 0.12^{c}	60.00 ± 16.33^{b}	6.02 ± 0.04^{d}
T3	7.03 ± 0.17^{d}	68.40 ± 0.29^{e}	17.95 ± 0.11^{d}	8.69 ± 0.03^{d}	2.30 ± 0.08^{d}	3.40 ± 0.08^{d}	66.67 ± 16.33^{b}	6.72 ± 0.14^{e}
T4	7.07 ± 0.05^{d}	68.80 ± 0.33^{e}	18.07 ± 0.09^{d}	8.59 ± 0.42^{d}	2.27 ± 0.17^{d}	$3.30 \pm 0.22^{d,e}$	66.67 ± 9.43^{b}	6.83 ± 0.08^{e}
T5	$\mathbf{7.90 \pm 0.08^{e}}$	$\mathbf{79.43 \pm 0.50^{f}}$	$\mathbf{18.56 \pm 0.03^{e}}$	$\mathbf{9.24 \pm 0.09^{e}}$	$\mathbf{3.03 \pm 0.17^{e}}$	$\mathbf{4.43 \pm 0.12^{f}}$	$\mathbf{73.33 \pm 9.43^{b}}$	$\mathbf{8.33 \pm 0.19^{g}}$
T6	$\mathbf{7.77 \pm 0.12^{e}}$	$\mathbf{79.53 \pm 1.31^{f}}$	$\mathbf{18.39 \pm 0.09^{e}}$	$\mathbf{9.17 \pm 0.09^{e}}$	$\mathbf{2.90 \pm 0.08^{e}}$	$\mathbf{4.23 \pm 0.21^{f}}$	$\mathbf{73.33 \pm 9.43^{b}}$	$\mathbf{8.02 \pm 0.09^{f}}$
T7	6.10 ± 0.16^{c}	47.63 ± 2.58^{c}	15.16 ± 0.08^{b}	7.46 ± 0.07^{b}	$2.17 \pm 0.12^{c,d}$	$2.83 \pm 0.21^{b,c}$	60.00 ± 16.33^{b}	5.47 ± 0.07^{c}
T8	6.23 ± 0.05^{c}	$49.67 \pm 1.19^{c,d}$	15.89 ± 0.16^{c}	7.60 ± 0.01^{c}	$2.17 \pm 0.17^{c,d}$	$2.97 \pm 0.12^{c,d}$	60.00 ± 16.33^{b}	5.57 ± 0.08^{c}
T9	2.83 ± 0.25^{a}	22.33 ± 1.33^{a}	13.80 ± 0.12^{a}	4.96 ± 0.10^{a}	1.33 ± 0.12^{a}	1.63 ± 0.12^{a}	26.67 ± 9.43^{a}	3.44 ± 0.15^{a}

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values ($P < 0.05$). T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control

**data means of three pots*

Values in bold indicate the most effective treatment

Table. 17 Effect of EPS (at various concentrations) and selected isolates *Pseudomonas entomophila* PE3 and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on biochemical parameters of sunflower under saline conditions (pot study)

Treatments	Total chlorophyll content (mg/ gm)	Carotenoid content (mg/ gm)	Flavonoid content (µg/ gm)	Phenol content (µg/ mg)	Protein content (mg/ gm)	Carbohydrate content (mg/ gm)
T1	9.38 ± 0.06^{b}	0.21 ± 0.001^{b}	75.53 ± 0.58b	200.07 ± 7.20^{b}	12.97 ± 0.18^{b}	283.73 ± 6.70^{b}
T2	9.86 ± 0.07^{d}	0.23 ± 0.001^{c}	77.22 ± 0.70b	217.93 ± 0.57^{c}	13.85 ± 0.14^{e}	305.87 ± 3.58^{c}
T3	11.30 ± 0.09^{e}	0.25 ± 0.001^{d}	82.38 ± 0.67c	248.87 ± 3.55^{d}	15.40 ± 0.18^{f}	418.40 ± 10.48^{d}
T4	11.29 ± 0.07^{e}	0.25 ± 0.002^{d}	82.56 ± 0.55c	247.67 ± 3.58^{d}	15.36 ± 0.10^{f}	419.20 ± 6.21^{d}
T5	**12.69 ± 0.05^{g}**	**0.28 ± 0.002^{e}**	**101.60 ± 0.83d**	**289.53 ± 2.78^{e}**	**17.14 ± 0.11^{g}**	**640.53 ± 7.17^{f}**
T6	**12.45 ± 0.05^{f}**	**0.28 ± 0.002^{e}**	**100.89 ± 1.70d**	**286.27 ± 4.07^{e}**	**16.90 ± 0.07^{g}**	**619.07 ± 11.92^{e}**
T7	9.63 ± 0.05^{c}	0.22 ± 0.002^{c}	75.88 ± 0.33b	222.93 ± 5.59^{c}	13.27 ± 0.08^{c}	299.60 ± 4.28^{c}
T8	$9.75 \pm 0.02^{c,d}$	0.23 ± 0.001^{c}	76.95 ± 0.98b	224.93 ± 5.59^{c}	13.57 ± 0.09^{d}	303.20 ± 8.09^{c}
T9	7.84 ± 0.04^{a}	0.16 ± 0.003^{a}	68.32 ± 2.08a	161.07 ± 2.66^{a}	9.69 ± 0.12^{a}	110.80 ± 1.18^{a}

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values (P < 0.05). T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control

Values in bold indicate the most effective treatment

Table. 18 Effect of EPS (at various concentrations) selected isolates *Pseudomonas entomophila* PE3 and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on salt-tolerance traits of sunflower under saline conditions (pot study)

Treatments	Proline content (µg/ gm)	Reducing power (µg/ mg)	Antioxidant activity (% of DPPH inhibition)	Hydroxyl scavenging activity (% of inhibition)	Relative water content (%)	Electrolyte leakage index	Membrane stability index
T1	361.65 ± 3.29^{b}	40.39 ± 0.71^{b}	25.49 ± 0.36^{b}	32.30 ± 1.10^{b}	78.83 ± 0.15^{b}	62.61 ± 0.12^{f}	37.26 ± 0.09^{b}
T2	373.02 ± 2.28^{c}	42.23 ± 0.41^{c}	28.84 ± 0.25^{c}	35.47 ± 0.53^{d}	82.10 ± 0.09^{d}	49.93 ± 0.24^{d}	50.32 ± 0.09^{d}
T3	416.46 ± 3.92^{d}	51.39 ± 0.22^{d}	44.73 ± 0.92^{d}	47.47 ± 0.95^{e}	85.81 ± 0.04^{e}	41.30 ± 0.32^{c}	58.23 ± 0.07^{e}
T4	418.12 ± 1.05^{d}	51.32 ± 0.47^{d}	45.26 ± 0.86^{d}	47.40 ± 1.10^{e}	85.88 ± 0.03^{e}	42.48 ± 0.10^{c}	58.01 ± 0.45^{e}
T5	**466.39 ± 3.04^{e}**	**70.07 ± 0.51^{f}**	**53.39 ± 0.47^{e}**	**57.77 ± 0.69^{f}**	**96.65 ± 0.05^{g}**	**38.26 ± 0.09^{a}**	**61.38 ± 0.31^{g}**
T6	**462.88 ± 2.39^{e}**	**67.65 ± 0.65^{e}**	**52.49 ± 0.42^{e}**	**57.27 ± 0.82^{f}**	**94.10 ± 0.04^{f}**	**39.39 ± 0.11^{b}**	**60.65 ± 0.31^{f}**
T7	366.33 ± 3.04^{b}	41.59 ± 0.18^{c}	29.21 ± 0.65^{c}	$33.63 \pm 0.71^{b,c}$	80.22 ± 0.06^{c}	52.69 ± 0.13^{e}	47.32 ± 0.08^{c}
T8	$368.81 \pm 2.1^{b,c}$	41.63 ± 0.38^{c}	30.02 ± 0.50^{c}	$34.90 \pm 0.22^{c,d}$	80.84 ± 0.06^{c}	52.62 ± 0.11^{e}	47.58 ± 0.04^{c}
T9	133.80 ± 3.55^{a}	26.55 ± 0.48^{a}	22.88 ± 0.47^{a}	20.86 ± 0.20^{a}	57.41 ± 0.06^{a}	78.85 ± 0.09^{g}	21.12 ± 0.07^{a}

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values ($P < 0.05$). T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control

Values in bold indicate the most effective treatment

Table. 19 Effect of EPS (at various concentrations) and selected isolates *Pseudomonas entomophila* PE3 and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on growth parameters and productivity of sunflower under saline conditions (field study)

Treatments	Root length (cm)	Shoot length (cm)	Fresh weight (gm)	Dry weight (gm)	Germination rate* (%)	Stem girth (cm)	Head size (cm)	Seeds per plant	Weight of seeds per plant (gm)	Oil content (%)
T1	12.31 ± 0.48^{b}	88.45 ± 1.9^{b}	179.23 ± 1.7^{b}	127.11 ± 1.7^{b}	60.90 ± 0.04^{b}	4.49 ± 0.07^{b}	10.23 ± 0.11^{b}	717 ± 4.0^{b}	35.85 ± 0.20^{b}	34.02 ± 0.10^{b}
T2	13.01 ± 0.16^{c}	91.59 ± 1.0^{b}	200.11 ± 2.8^{c}	150.89 ± 1.5^{d}	74.58 ± 0.05^{e}	5.01 ± 0.07^{c}	11.02 ± 0.34^{c}	769 ± 4.0^{d}	38.45 ± 0.19^{c}	35.69 ± 0.74^{c}
T3	14.12 ± 0.34^{d}	100.53 ± 1.1^{c}	268.40 ± 1.1^{d}	209.23 ± 1.3^{e}	77.68 ± 0.14^{f}	6.39 ± 0.05^{d}	12.11 ± 0.08^{d}	839 ± 3.0^{e}	41.93 ± 0.15^{d}	39.61 ± 0.37^{d}
T4	14.26 ± 0.42^{d}	101.52 ± 1.0^{c}	270.37 ± 1.1^{d}	211.07 ± 1.4^{e}	$78.17 \pm 0.62^{f,g}$	6.40 ± 0.04^{d}	12.17 ± 0.04^{d}	844 ± 2.0^{e}	42.18 ± 0.11^{d}	39.66 ± 0.31^{d}
T5	**14.87 ± 0.25^{f}**	**114.44 ± 4.1^{e}**	**322.12 ± 5.7^{e}**	**266.17 ± 2.9^{e}**	**80.49 ± 0.94^{g}**	**7.17 ± 0.16^{e}**	**13.17 ± 0.11^{e}**	**982 ± 5.0^{f}**	**49.11 ± 0.26^{e}**	**41.02 ± 0.69^{e}**
T6	**$14.67 \pm 0.33^{d,e}$**	**107.22 ± 1.47^{d}**	**316.14 ± 5.50^{e}**	**262.40 ± 6.87^{f}**	**80.79 ± 0.86^{h}**	**6.45 ± 0.10^{d}**	**12.94 ± 0.20^{e}**	**976 ± 5.72^{f}**	**48.80 ± 0.29e**	**$40.31 \pm 0.29^{d,e}$**
T7	$12.55 \pm 0.16^{b,c}$	89.94 ± 3.92^{b}	183.72 ± 2.54^{b}	$132.33 \pm 4.55^{b,c}$	66.61 ± 0.98^{c}	4.60 ± 0.06^{b}	10.10 ± 0.15^{b}	728.33 ± 7.13^{c}	36.45 ± 0.82b	36.45 ± 0.82^{b}
T8	$12.60 \pm 0.25^{b,c}$	89.40 ± 1.3^{b}	184.62 ± 3.5^{b}	139.57 ± 1.4^{c}	69.68 ± 0.49^{d}	$4.76 \pm 0.11^{b,c}$	10.41 ± 0.29^{b}	732 ± 2.0^{c}	36.62 ± 0.11^{b}	$34.78 \pm 0.24^{b,c}$
T9	10.01 ± 0.22^{a}	61.93 ± 1.4^{a}	84.43 ± 3.2^{a}	64.46 ± 1.8^{a}	30.41 ± 0.18^{a}	2.29 ± 0.12^{a}	3.65 ± 0.22^{a}	416 ± 4.0^{a}	20.79 ± 0.18^{a}	20.41 ± 0.25^{a}

*Data are means of ten replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values ($P < 0.05$). T1- 0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control. *data means of three blocks*

Values in bold indicate the most effective treatment

Table. 20 Effect of EPS (at various concentrations) and selected isolates *Pseudomonas entomophila* PE3 and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on biochemical parameters of sunflower under saline conditions (field study)

Treatments	Total chlorophyll content (mg/ gm)	Carotenoid content (mg/ gm)	Flavonoid content (µg/gm)	Phenolic content (µg/mg)	Carbohydrate content (mg/ gm)	Protein Content (mg/ gm)
T1	16.31 ± 0.05^{b}	0.207 ± 0.002^{b}	111.21 ± 0.77^{b}	183.53 ± 2.5^{b}	468.40 ± 5.94^{b}	19.85 ± 0.11^{b}
T2	17.73 ± 0.08^{d}	0.228 ± 0.001^{d}	121.26 ± 0.86^{c}	213.93 ± 5.0^{d}	656.93 ± 1.79^{e}	22.61 ± 0.13^{d}
T3	20.07 ± 0.10^{e}	0.274 ± 0.001^{e}	128.82 ± 0.42^{d}	279.80 ± 1.8^{e}	785.73 ± 8.76^{f}	28.28 ± 0.07^{e}
T4	20.26 ± 0.16^{e}	$0.276 \pm 0.001^{e,f}$	129.98 ± 1.09^{d}	282.80 ± 2.0^{e}	793.33 ± 2.98^{f}	28.70 ± 0.20^{e}
T5	**21.02 ± 0.13^{f}**	**0.286 ± 0.002^{f}**	**142.08 ± 2.10^{f}**	**328.13 ± 4.7^{g}**	**844.93 ± 12.91^{h}**	**29.95 ± 0.28^{g}**
T6	**20.96 ± 0.13^{f}**	**0.283 ± 0.002^{f}**	**135.59 ± 4.29^{e}**	**309.2 ± 4.8^{f}**	**828.8 ± 13.25^{g}**	**$29.14 \pm 0.23^{e,f}$**
T7	16.50 ± 0.16^{b}	$0.212 \pm 0.002^{b,c}$	114.68 ± 2.19^{b}	$189.5 \pm 1.6^{b,c}$	612.93 ± 9.48^{c}	20.49 ± 1.18b,c
T8	17.14 ± 0.10^{c}	0.216 ± 0.001^{c}	115.12 ± 1.66^{b}	192.07 ± 2.4^{c}	629.87 ± 1.64^{d}	21.30 ± 0.24^{c}
T9	6.86 ± 0.09^{a}	0.108 ± 0.001^{a}	89.94 ± 0.92^{a}	132.93 ± 1.9^{a}	162.80 ± 1.20^{a}	16.74 ± 0.12^{a}

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values (P < 0.05). T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control

Values in bold indicate the most effective treatment

Table. 21 Effect of EPS (at various concentrations) and selected isolates *Pseudomonas entomophila PE3* and *Alcaligenes faecalis* AF7 (alone and in combination with EPS) on salt-tolerance traits of sunflower under saline conditions (field study)

Treatments	Proline content (µg/gm)	Reducing power (µg/mg)	Antioxidant activity (%)	Hydroxyl scavenging activity (%)
T1	477.08 ± 1.62^{b}	73.59 ± 0.38^{b}	36.80 ± 0.58^{b}	48.40 ± 2.03^{b}
T2	564.33 ± 5.60^{c}	78.39 ± 0.23^{c}	50.50 ± 0.48^{d}	59.57 ± 1.47^{d}
T3	669.58 ± 9.61^{d}	86.01 ± 0.36^{d}	54.49 ± 0.58^{e}	62.83 ± 1.43^{e}
T4	672.50 ± 2.30^{d}	$87.29 \pm 0.87^{d,e}$	55.10 ± 0.49^{e}	64.07 ± 1.05^{e}
T5	**682.42 ± 2.77^{e}**	**88.07 ± 1.01^{f}**	**60.82 ± 1.01^{f}**	**70.18 ± 1.74^{g}**
T6	**673.52 ± 2.81^{d}**	**87.87 ± 0.54^{e}**	**59.53 ± 1.28^{f}**	**67.2 ± 1.3^{f}**
T7	484.74 ± 1.01^{b}	74.06 ± 1.40^{b}	45.75 ± 0.69^{c}	48.6 ± 0.9^{b}
T8	483.08 ± 1.25^{b}	77.01 ± 0.38^{c}	47.24 ± 0.64^{c}	50.78 ± 1.72^{c}
T9	251.00 ± 1.83^{a}	49.29 ± 0.18^{a}	28.94 ± 0.54^{a}	20.17 ± 1.26^{a}

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values ($P < 0.05$). T1-0.1% EPS, T2- 1.0% EPS, T3-2.0% EPS, T4-5.0% EPS, T5-2% EPS+PE3, T6- 2% EPS + AF7, T7-AF7 only, T8-PE3 only, T9- untreated control
Values in bold indicate the most effective treatment

DISCUSSION

The increasing trend of pollution and climate change has entirely changed the land use systems thereby elevating level of contaminated soils. Along with the increase in contaminated soil there is found parallel increment in saline soils, pertaining to the involvement of human activities in every niche and ecosystem on earth. The continuous increase in soil salt level along with unchecked erosion by wind and water demands a cost effective, sustainable and large scale applicable strategy to remediate the lands in limited time. Although the application of chemicals is highly effective in treating salty soils but because of the xenobiotic or non-ecofriendly nature they further threaten the fertility of land (Artiola et al. 2019). Thus, PGPB are the imperative call in popularizing the concept of organic farming and a sustainable remediation technique to ameliorate the saline-sodic soils. PGPB are not only involved in defining the health status of plants but are also the key players in framing the structure and fertility of eroded lands such as saline or sodic soils (Arora and Vanza 2017).

The increasing saline-agricultural lands have threatened the productivity of soil and has reduced the quality of crops. Salinity poses oxidative and ionic stresses in plants affecting their physiological and metabolic processes (Flowers and Colmer 2008). Plants are first in the food chain to get effected by salinity and resulting in overall affect on the food availability (Vaishnav et al. 2016). Matilla et al. (2007) reports that there are mainly two selective forces that aid colonization of microbes in rhizosphere including stress adaption and availability of specific nutrients. Therefore, the colonization and selection of ST-PGPR is an important strategy of plants to mitigate the ill-effects of salinity.

In the present study, semi-arid region of Kanpur Dehat (UP, India) was selected as the sampling site. Soil of this region is categorized as highly saline with EC >16 dS/m (National soil survey handbook;

http://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/ref/?cid=nrcs142p2_054242) with relatively low nutrient content and organic matter. In total 42 salt-tolerant bacteria were isolated and screened on the basis of salt-tolerance and plant growth promotion activities. The phenotypic characterization showed that majority of isolates were pigmented and mucilaginous, showing fluorescence under UV light and also were fast growers. Jida and Asefa (2011) characterized that isolates with generation time between 1-3 hours are fast growers, while generation time more than 4 hours are slow growers. Lamichhane and Varvaro (2013) discussed that production of fluorescent pigments (mainly siderophores) are the major cause of fluorescence reported in bacteria especially those belonging to fluorescent pseudomonads group. Examining the tolerance capacity of isolates against gradients of pH and temperature it was found that the optimum temperature range was 25-30^{o}C while pH range was 6-10. Most of the isolates were inhibited at 45^{o}C and pH below 5. Šovljanski et al. (2021) also discussed the tolerance capacity of isolates obtained from alkaline soil and found that majority of the isolates (from alkaline soil) could not grow at 44^{o} C and the optimum temperature was around 30-37^{o}C. Further, the study confirmed pH as a critical parameter and only few isolates were able to grow in acidic pH (4), while pH between 9-11 was optimum for growth.

The two most potent isolates (selected on the basis of PGP properties and salt-tolerance levels) *i.e.*, PE3 and AF7 were subjected to salt–tolerance assay to determine the effect of salinity on bacterial growth. For both the isolates it was found that upto 2% NaCl condition, there was no effect on growth pattern. However, salinity above 4% reduced the growth rate and there was significant reduction at 8% NaCl. Comparing with earlier reports and criteria, isolate with ability to tolerate up to 8% NaCl can be categorized as moderately halotolerant bacterium (Ventosa et al. 1998; Rahman et al. 2017), therefore,

PE3 and AF7 can be considered as moderately halotolerant bacteria. There are previous reports about isolation of halotolerant bacteria from saline soil including the study of Rahman et al. (2017) showing halotolerant strains cultured from coastal Patenga area of Bangladesh with tolerance upto 6% NaCl (w/v). Similarly, Albdaiwi et al. (2019) isolated extremely halotolerant bacteria from saline areas near Dead Sea and reported their tolerance level even upto 25% NaCl.

Colonies of most of the isolates in the study were cream colored and mucilaginous, 62.2% were Gram negative, rod shaped while 37.8% were Gram positive and rod shaped. Classification and identification of bacteria should be based on overall phenotypic, biochemical and morphological pattern and a single characteristic is insufficient to determine the taxonomy (Baron 1996; Pitt and Barer 2012). Therefore, in the present study carbon and nitrogen utilization pattern along with various biochemical and enzymatic tests were conducted to characterize the isolates. The most preferred carbon source was glucose and nitrogen sources were yeast extract, KNO_3, $NaNO_3$, tryptophan and NH_4Cl and least growth was reported when medium was amended with malate (carbon source) and glycine (nitrogen source). The genus *Pseudomonas* has been reported to show nutritional versatility (Misko and Germida 2002; Silby et al. 2011). Further, Botelho and Mendonça-Hagler (2006) added that utilization of diverse sugars and amino acids help the microbes to consume root exudates and establish themselves in the rhizosphere aiding colonization. Jacoby et al. (2020) declared that ammonium is the most preferred nitrogen source for most of the bacteria and is often used as standard control however, soil bacteria exhibit extensive nitrogen substrate diversity and the associated metabolic pathways. Kramer et al. (2016) reported that glucose is mainly utilized by *Arthrobacter, Flavobacterium, Pseudomonas and Humicoccus.* The isolates were characterized as per the standards of

Bergey's Manual of Systematic Bacteriology (Garrity 2005). The production of different extracellular enzymes such as catalase, amylase, lipase, citrate, oxidase, cellulase, protease, pectinase by the isolates enable colonization of microbes and also are helpful in different biotechnological processes as well as for human health (Razzaq et al. 2019; Amna et al. 2019).

The selected salt-tolerant isolates were then subjected to proteomic and molecular identification techniques *i.e.* MALDI-TOF MS biotyping and 16S rRNA sequencing respectively. The selected PGP isolates PE3 and AF7 were identified as *Pseudomonas entomophila* and *Alcaligenes faecalis* respectively. The other isolates were found to be belonging to genera *Bacillus, Cellulomonas* and *Alcaligenes.* The 16S rRNA sequences of isolate PE3 (accession number MN515387) and AF7 (accession number MK898928) were submitted to GenBank. Both the isolates (PE3 and AF7) were also submitted to international culture collection repository at NAIMCC-Mau, India. Describing the essentiality of using two methods for identification of bacteria, it is suggested that the combination of MALDI-TOF MS and 16S rDNA sequencing allows identification of various bacterial species with more reliability and accuracy (Schröttner et al. 2016; Strejcek et al. 2018).

P. entomophila has previously been reported as salt-tolerant PGP strain with the potential to mitigate salinity stress in plants. Li et al. (2017) isolated for the first time nitrogen fixing *P. entomophila* CN11 strain from the rhizosphere of sugarcane with other potential PGP traits such as ACCD, IAA production and phosphate solubilization. The strain CN11 also exhibited osmotic stress tolerance. Ansari and Ahmad (2018) also found production of IAA, HCN, siderophore, EPS along with P solubilization traits in *P. entomophila* FAP1. Fathalla (2018) reported activities of EPS production and biofilm formation by *P. entomophila* strain under salinity stress.

Similarly, genus *Alcaligenes* has been previously reported with efficient salt-tolerating ability. Behera et al. (2015) reported a halophilic strain *A. faecalis* D-TSB-1 which was able to tolerate 15% NaCl. Egamberdieva et al. (2019) reported salt tolerant *A. faecalis* TSAU3 from rhizosphere of wheat growing in saline soil with EC value of 560 ± 61 ms m^{-1}. Similarly, Bal et al. (2013) reported halotolerant *Alcaligenes sp*. strain from the coastal rice fields with soil EC 6.88 dS/m.

Fluorescent pseudomonads are regarded as model organisms to study the interaction between plant and microbes and the resultant enhancement in growth and yield both under hospitable and unhospitable conditions (Sitaraman 2015). Analyzing various beneficial microbes, *Pseudomonas* genus is regarded as core of PGPR for growth promotion in various crops (Sutra et al. 2000). Qessaoui et al. (2019) discussed that *Pseudomonas* proportions were 10% in rhizospheric soil, 4.24% in rhizoplane and 9.72% in endorhizospheric component. The potential of pseudomonads in colonizing the root also make them potent candidates for bio-inoculant development (Samaddar et al. 2019). PGPR assign various mechanisms for growth promotion and inducing salt-tolerance in plants. The direct mechanism of action involves nutrient assimilation, phytohormone production, EPS synthesis and biofilm formation. Further to mitigate salt-toxicity in plants PGPR accumulate osmolyte/ osmoprotectants, produce ACCD enzymes for sinking of stress-ethylene, scavenge ROS through antioxidant systems immobilize salts thereby reducing their availability to plants (Arora et al. 2020a).

In the present study, the selected isolates (PE3 and AF7) were subjected to salinity gradients (2%-8% NaCl) and various PGP and salt-tolerance traits were examined to estimate the dominant responses in bacteria. Qualitative and quantitative analysis of PGP properties confirmed PE3 as IAA, GA, EPS and siderophore producer along with the ability to solubilize P, K and Zn and ACCD enzymatic activity both under saline

and non-saline conditions. Similarly, AF7 also showed PGP properties except K solubilization even under saline conditions. According to Tewari and Arora (2016) the maintenance of bacterial population (even under salt stress) could be the important factor for exhibition of PGP properties at high salinity. The availability of P to plants helps in root formation, protection against stresses, sugar accumulation, stomatal conductance, transpiration, seed germination, gain in plant biomass, water uptake and formation of adenosine triphosphate (ATP) (Prakash and Arora 2019). For both the isolates (PE3 and AF7) P solubilization was maintained upto 2% NaCl and then decreased with no assimilation at 8% NaCl. PE3 was a better P solubilizer under saline conditions while AF7 showed no chelation above 2% NaCl. Similarly, PE3 was found to be a potent Zn and K chelator upto 2% NaCl and higher saline conditions reduced the property. Mahajan et al. (2020) reported nutrient chelation ability of *Pseudomonas stutzeri* SGM-1 which was found tolerant even upto 12% NaCl and the strain was able to promote growth of chickpea upto 300 mM NaCl. Explaining the role of zinc in salt-mitigation, Kamran et al. (2017) concluded that the micronutrient is involved in carbohydrate metabolism and even act as antioxidant. K is also an important nutrient with significant role in salinity including reduction in ROS status of plants, decrease in the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and retaining the photosynthetic electron transport activity. K is also involved in carbohydrate metabolism, enzyme activation, cation-anion balance, osmoregulation, water uptake and growth and developmental processes (Wang and Wu 2013; Hasanuzzaman et al. 2018). Ansari et al. (2019a) showed that inoculation of *Medicago sativa* L. plants with *Pseudomonas* sp. mitigated salt stress in plants through enhanced K^+ uptake and reduced Na^+ accumulation.

The production of phytohormone IAA and GA by PE3 and AF7 increased at 2% NaCl as compared to non-saline control and the property was present even at 8% NaCl, however, there was significant reduction above 4% NaCl. Similar results were shown by Amna et al. (2019), suggesting that increase in salinity enhanced phytohormone production by *Bacillus* sp.. Yousef (2018) also found that IAA production by *B. subtilis* at 0.5% and 1% NaCl was higher in comparison to non-saline control and concluded that culture conditions and substrate variations may modify IAA productivity level. Upadhyay et al. (2009) reported that salt-tolerant stains of *Bacillus* produced GA but higher salt concentrations (8% NaCl) inhibited the production. IAA is directly involved in development of roots and altering their structures for better stress adaptation (Abbas et al. 2018). Gibberellins are important for cell elongation and division, root, stem and leaf extension, seed germination or dormancy, fruit senescence and flowering (Miceli et al. 2019). Corroborating with the present results, Shultana et al. (2020) found that salt-tolerant strains of *Bacillus tequilensis* and *Bacillus aryabhattai* produced IAA and solubilized phosphate at all salinity levels (0-2 M NaCl). Paul et al. (2014) found that when the salinity was increased to 300 mM NaCl (almost equivalent to 2% NaCl) the IAA production by *Azotobacter* strain increased while P solubilization activity decreased.

Isolate AF7 and PE3 also managed to produce siderophore up to 4% NaCl, but higher salinity inhibited the production. Through siderophore production PGPR help the plant to obtain iron from the soil and protect them from phytopathogens. Chelation of iron helps in regulating various metabolic processes in plants such as chlorophyll synthesis, electron transfer in photosynthetic and respiratory chain processes and also aids in synthesis of DNA, RNA and their repair (Ferreira et al. 2019). Mudhulkar et al. (2018) identified a novel siderophore Acinetoamonabactin which was produced by halo-

tolerant strain of *Acinetobacter soli* and was able to chelate iron with high affinity under iron-starvation conditions such as that of saline soils. Genus *Pseudomonas* is already designated as efficient siderophore producer with various class of siderophores produced under neutral and alkaline conditions and imparting yellowish-green pigment to bacteria (Cornelis and Matthijs 2007; Ringel and Bruiser 2018). Vindeirinho et al. (2020) elaborated that production of siderophore pyoverdin by *P. fluorescence* was manipulated by modifying the composition of medium or changing the medium from King's B to succinate minimal salt medium. Correlating with the work, this study also reported that when the culture medium was supplemented with 2% NaCl, the siderophore production by both the isolates PE3 and AF7 increased.

Further, to mitigate salt-stress in plants, beneficial microbes lower the ethylene level *via* production of ACC deaminase which hydrolyses ACC to α-ketoglutaraldehyde along with supplementation of energy (Gamalero and Glick 2015). Characterizing the selected isolates for qualitative estimation of ACC deaminase activity, it was found that both PE3 and AF7 were able to grow on plates with ACC as sole N source, confirming the activity of enzyme. ACC deaminase activity has been elucidated as selective criterion for survival of bacteria under stressed conditions. Timmusk et al. (2011) found that 50% of bacterial population isolated from South Facing Slope in Northern Israel, where the climatic conditions were adverse with excessive sunlight and frequent drought, had ACC deaminase activity whereas in Northern Israel where the climatic conditions were hospitable only 4% exhibited the activity. Qi et al. (2021) co-inoculated salt-stressed cucumber seedling with plant growth promoting (mainly nitrogen fixing) *B. subtilis* NX-62 with salt-tolerant ACC deaminase producing *B. licheniformis* NX-3 and *B. subtilis* NX-4. The results showed that co-inoculation was better than sole and

non-inoculation with significant increase in growth parameters and nutrient status of plants.

EPS produced by PGPR, particularly under saline conditions, play an important role in survival of microbes and plants as well as support their growth and yield (Mukhtar et al. 2020). Confirming the concept that EPS production is the most important and primary response of bacteria against salinity stress (Khan et al. 2019), the present study illustrated that there was approximately threefold increase in EPS production by PE3 and AF7 from non-saline condition to 2% NaCl salinity. Fathalla (2018) explained that with increase in salinity, EPS production increased as compared to non-saline control. It has been reported that in some PGPB, particularly those obtained from saline soils, production of EPS increases up to certain levels of salinity, suggesting its role in cell protection (Egamberdieva et al. 2019). Zeng et al. (2016) reported salinity as an important factor for EPS production. The study found that EPS production by *Rhodopseudomonas acidophila* increased with change in NaCl concentration from 0 to 10.0 g/L, and the metabolite was the main reason for increase in microbial biomass and cell survival. Yaakop et al. (2016) also observed elevated expression of EPS gene clusters at 20% (w/v) NaCl. Kasotia et al. (2016) concluded that the amount of EPS produced by *Pseudomonas* strain AK1 increased from 0 mM to 300 mM NaCl and with the increase in EPS production, the EC_e of the inoculated plants decreased to 0.9 dS/m due to the binding of Na^+ to EPS. Dixit et al. (2020) reported alkalotolerant strain of *A. faecalis* NBRI NB2.5 with the ability to produce IAA, EPS and solubilize P but no siderophore production.

The onset of salinity stress invokes various synergistic mechanisms in bacteria including osmolyte accumulation, mitigation of ROS through antioxidant systems, ion homeostasis, biofilm formation and EPS production, which help in the survival and

protection against hyperosmotic surroundings (Pan et al. 2019; Arora et al. 2020a). Therefore, to estimate the exact response in the isolates (PE3 and AF7), salt-tolerance properties were checked. The most important concept of salinity amelioration involves the decreased availability of Na^+ to plants and regulating osmotic homeostasis to deduce the chances of flaccidity and death (Isayenkov and Maathuis 2019). Studying the ability of PE3 and AF7 to accumulate Na^+, it was found that upto 6% NaCl there was continuous increase in sodium accumulation and then at 8% NaCl the amount although decreased yet it was significantly higher as compared to non-saline condition. There was a direct relationship established between salinity and sodium accumulation by bacteria which could possibly induce toxicity in microbes. However, according to Assaha et al. (2017) along with sodium accumulation, microbes initiate counter mechanisms of creating many vacuolar structures for influx of cytoplasmic sodium ion through different porters. Also the simultaneous accumulation of soluble sugars or proteins referred as osmoprotectants by salt-tolerant microbes balance the osmotic difference and dilute the salt-toxicity (Weinisch et al. 2018). Similar concept and mechanism was also found in the present study where along with accumulation of sodium the isolates also showed higher fold synthesis of osmoprotectant proline with increase in salinity. These results are in corroboration with the study of Giri et al. (2013) which showed enhanced Na^+ accumulation by salt-tolerant strains of *Methylophilus* sp. and *Methylobacterium* sp.. Apart from osmotic balancing, proline synthesis also regulates hydroxyl radical scavenging and reduced enzymatic inactivation and after the episode of stress is over it provides energy to the plant/ microbe for driving essential metabolic activities (Wang et al. 2018). Therefore, concluding from the statistical analysis and the mathematical model it can be summarized that at lower levels of

salinity (i.e., up to 2% NaCl) PGP properties were dominant, while at higher (i.e., above 4% NaCl) the stress adaption and survival responses were prominent.

Biofilming is an important attribute of bacteria under salinity stress which provides protection against external stresses and aid in plant-microbial interactions. Biofilm is an aggregate of microbes adhered to one another protecting against inhospitable conditions (Kumar et al. 2020).The formation of biofilm is regulated by various signalling processes and production of EPS which holds the assembly of microbial colonies (Rode et al. 2020; Bhagat et al. 2021). Confining to the objective of the study (fluorescent pseudomonads), PE3 was selected for biofilm activity (using SEM and ILSCM). In the present study through colorimetric assay, biofilm formation was increasing with salinity upto 4% NaCl and then reduced with least production at 8% NaCl. Confirming the results through confocal microscopy (for PE3) it was found that biofilm formation on glass surface was more at 2%, 4% and 6% NaCl, lesser under non-saline and almost negligible at 8% NaCl. The density of biofilm was found maximum at 4% NaCl as observed through Z-stack analysis. Elongated bacterial cells were seen at 6% NaCl; explaining the mechanism Noirot-Gros et al. (2018) concluded that exposure to stresses leads to transient formation of elongated cells. EPS matrix visible in the images proved the role of biomolecule in adhesion and cohesion. Drogue et al. (2013) discussed the role of EPS in cohesion of bacterial cells and adhesion of biofilm to root or solid surfaces enabling exchange of signals, nutrients and water inside a stable protected micro-environment. Wang et al. (2019) reported biofilm formation by *B. amyloliquefaciens* 54 which protected the tomato plants from drought stress through increase in survival rate, root vigor, RWC, activity of antioxidant enzymes and expression of stress-responsive genes like *ltpg2, tdi65,* and lea. The study also elaborated the role of biofilm formation by inoculating the tomato plants with mutants

capable of hyper-robust biofilm formation (*ΔabrB* and *ΔywcC*) and found that drought resistance was increased; contrarily plants inoculated with mutants defective of biofilm formation (*ΔepsA-O* and *ΔtasA*) showed no growth under water deficit conditions.

Biofilming in the rhizosphere is an important attribute for not only protection against various biotic and abiotic stresses but also encourages root exudation thereby increasing the chances of bacterial colonization. Biofilming even catalyzes the processes of biodegradation of organic pollutants and heavy metals (Pandit et al. 2020). Studying the biofilming activity by PE3 on sunflower root surface under laboratory conditions, it was found that the bacteria was able to colonize the sunflower root and the colonies were surrounded with some sort of protective matrix *i.e.,* biofilm. EPS further aided the activity and the cell number was increased on the root surface upon the addition of metabolite. Noirot-Gros et al. (2018) observed similar colonization patterns and biofilm matrix and illustrated that *Pseudomonas* strains SBW25, Pf0-1, Pf-5, and WH6 formed dense-biofilm structures on Aspen roots with colonies encased in mucilaginous matrix. de Zélicourt et al. (2018) showed increased colonization of Arabidopsis roots (analyzed through confocal microscopy) by *Enterobacter* sp. SA187 when the roots were exposed to 100 mM NaCl. Arora et al. (2020a) explained that the establishment of microbial biofilm regulate organic matter, ensure coagulation of soil particles leading to humus formation and thereby reviving the fertility of marginal saline soils.

Elucidating the changes in EPS (extracted from PE3) upon exposure to salinity, the present study highlighted that there was increase in sugar, protein and phenolic content of EPS under salt stress with maximum at 2% NaCl. Berne et al. (2015) elucidated that properties of each polymer are dependent upon their composition. Fang et al. (2018) correlated that changes in carbohydrates and protein content of EPS substantially modifies the functional and structural groups, hydrophobicity, surface charges,

flocculation rate and metal ion composition of bacteria. The study further confirmed that salinity is the key factor for inducing changes in EPS composition. There are previous reports signifying the importance of EPS components in imparting salt tolerance in the synthesizing microbe. According to Sandhya et al. (2009) carbon reservoir of EPS protects the microbes and plants under abiotic stresses and also enhances the water potential and regulates osmotic balance. The protein content of EPS induces membrane stability and protects the cell by providing osmotic balance under saline conditions (De Lacerda et al. 2003; Kavi Kishor et al. 2005). The phenolic content of EPS mediates seed germination, improve plant metabolism and stimulate the stress tolerance properties (Sharma et al. 2019).

In this study, there were unique observations regarding the changes in EPS functional groups with increase in salinity stress as observed through FT-IR analysis. An interesting finding is that the acetyl-group of uronic acid was only found in EPS obtained from non-stressed PE3 cells and absent in EPS extracted under saline conditions. Acetyl groups of uronic acid are previously reported as site for sodium ion binding (Mukherjee et al. 2019). Therefore, the absence of this entire band (in salinized EPS) indicates the accumulation of sodium by EPS. In addition, Gutierrez et al. (2013) reported that uronic acid configure polyanionic (negative charge) nature to EPS. This in turn is significant for microbial adhesion and biofilm formation, emulsification of hydrophobic compounds such as oils and stimulating their degradation. Further, Hassler et al. (2011) reported chelation of Fe^{+3} by acetyl group which is important for plant growth and soil reclamation. The free functional groups found in both salinized (at all salt-conditions) and non-salinized EPS (present study) including carboxyl, amide and phosphoryl are previously discussed as important for causing soil aggregation and chelation of cations such as Ca^{+2} , Zn^{+2} , Mg^{+2} (Yi et al. 2008).

Further confirming the configuration of EPS, GC-MS was conducted and it was found that the monomeric unit of the polymer was unusual trisaccharide *i.e.* melezitose. The novel character of the EPS was highlighted (to the best of our knowledge) as the sugar is reported for the first time in any bacterial polysaccharide. The quantity of the sugar was maximum at 2% NaCl and then decreased corresponding to the colorimetric assay conducted in the study. Additional sugars (β-D-glucose and β-D-galactose) were also present in EPS extracted at 2% NaCl and guanosine was found in all EPS except that produced at 8% NaCl. The non-carbohydrate unit of EPS consisted of protein, esters (fatty acids) and carboxylic acid and the esters were increasing with salinity. The presence of different moieties in EPS suggested that the polymer is a heteropolysaccharide and 2% NaCl was mainly triggering the compositional changes in the molecule. Similarly, Chikkanna et al. (2018) also reported that EPS from halophile *Halomonas nitroreducens* strain WB1 was composed of carbohydrates, proteins, acetyls, pyruvic acid, and hexosamines just like in the present study.

EPS are directly involved in formation of organo-mineral sheath important for soil aggregation especially in saline soils. Liu et al. (2013) highlighted that EPS from *B. subtilis* enriched in lipids and proteins was effective in adsorbing soil minerals (goethite). C, N and P fractions of *P. putida* X4 EPS had higher mineral absorption capacity due to electrostatic interactions between charged groups and minerals kaolinite, montmorillonite, and goethite (Lin et al. 2016; Costa et al. 2018). Redmile-Gordon et al. (2020) elaborated that EPS-protein are more associated with soil aggregate formation and soil-stability than EPS-sugars. Verdugo (2012) elucidated that EPS from marine microbes are important for surface attachments and cell aggregation and relatively the hydrophobic interactions supported by sugars along with proteins (of EPS) influence the phenomenon. Casillo et al. (2018) report that carboxylic acids and

sulphate groups in EPS impart sticky nature to the polymer. Therefore, comparing the composition of PE3-EPS it can be summarized that the polymer produced at higher salinity (mainly at 2% and 4% NaCl) elevate the carbohydrate content to ensure better microbial survival under stress and the presence of proteins and fatty acids (esters/ lipids) enable hydrophobic interactions to chelate cations and support biofilm formation and emulsification. Kumar et al. (2020) showed that polysaccharide-lipids are responsible for desiccation tolerance under abiotic stresses such as salinity and drought. The XRD analysis of EPS at various saline conditions revealed semi-amorphous nature of the biopolymer and the degree of crystallinity as well as the size of crystallite was increasing with salinity upto 4% NaCl. The diffractogram clearly showed the presence of NaCl in EPS which was irreversibly trapped in the biopolymer due to electrostatic interactions. However, under non-saline condition the EPS showed amorphous nature. To the best of our knowledge, till date there are no studies to determine the effect of NaCl on EPS through XRD analysis. Mosharaf et al. (2018) also found broad peaks in the diffractogram of EPS through XRD analysis and characterized the polymer as amorphous in nature. Mathivanan et al. (2021) reported amorphous nature of EPS under control and metal exposed condition. Interestingly, similar to the trapping of NaCl in the present study, Mathivanan et al. (2021) showed transformation of metals and their precipitation in EPS. Mukherjee et al. (2019) also obtained major characteristic diffracting peaks at 32.1 and 45.6 Å (also in PE3 EPS at 2-8% NaCl) in XRD analysis of *Halomonas* sp. Exo1 EPS under saline conditions.

SEM analysis of EPS revealed that there were significant changes in the morphology upon exposure to salinity. According to Kanamarlapudi and Muddada (2017) the difference in morphology and topography of EPS is mainly induced by changes in sample extraction, preparation, and purification and in the present study the

modification factor was salinity. The agglomerations found in non-salinized EPS mainly shows the presence of glycosidic bonds capable of forming gels (Chambi et al. 2021). EPS extracted at 2% NaCl showed most significant changes with smooth and rigid surface. More rigidity could possibly be to initiate shielding mechanism against increasing osmolarity, thereby protecting the microbes from hyper-osmotic environment. Relatively, bacteria increase membrane rigidity against polluted environment to inhibit entry of toxicants. EPS are major constituents of surface biofilms or outer matrix which decide the shape and rigidity of cells (Iyer et al. 2005; Murínová and Dercová, 2014). Mathivanan et al. (2021) reported that under metal amended conditions the structure of EPS appeared to be smooth and rigid facilitating the cell wall protection against metal toxicity. There are no related work to show the topographical changes in EPS upon exposure to salinity and the unique trapping mechanism found in the PE3-EPS.

EDX analysis showed the presence of C, O, Na and Cl and the elemental composition of EPS was changing with increase in salinity. C and O have been previously reported as major constituents of EPS (Boukhelata et al. 2019; Amer et al. 2021; Gangalla et al. 2021). The presence of osmolytes Na and Cl with varying concentrations was mainly due to their accumulation by EPS and the presence of salt crystals were also confirmed in SEM analysis. Mukherjee et al. (2019) also showed the presence of osmolytes including Na, Cl, Mg, Ca, P, S along with major ratios of C, N, and O.

This study further proved that EPS (extracted from PE3; as per the objectives of the study) is a potential reducing agent, antioxidant, sodium accumulator, water and nutrient chelator, biofilming agent and an emulsifier. The structural details showing the presence of functional groups and charged moieties may be the reasons for salt stress amelioration properties of EPS. Kanamarlapudi and Muddada (2017) reported that

Streptococcus thermophilus extracted EPS possessed antioxidant, hydrogen peroxide activity, reducing power and emulsification potential, but in the absence of salinity. Abdhul et al. (2014) found that bacterial EPS inhibited DPPH, superoxide and hydroxyl radicals by 63.5, 77.3 and 38.4 respectively (at conc. 8 mg/mL) and was increasing along with the concentration of free radicals. However, PE3-EPS was comparatively a better ROS scavenger even at lower concentration. Sun et al. (2020) also reported moderate antioxidant and emulsifying activity in EPS extracted from *Pantoea alhagi* and was composed of glucose, galactose, glucuronic acid, glucosamine and mannose. Similarly, Ansari et al. (2021) highlighted that novel biofilming strain *Pseudomonas azotoformans* FAP_5 produced EPS even under higher level of water stress and protected the bacterial cells from dehydration through formation of biofilms. Lami et al. (2021) also reported salt-tolerance in *P. stutzeri* strain MJL19 mainly due to the involvement of atleast three types of EPS: Psl-like, cellulose and alginate and the study suggested that components of EPS (sugars/ proteins) were modified due to salinity. The study also elaborated that EPS trapped ions from rhizosphere and thereby maintained homeostasis in soybean plants as well as microbes.

Further in the present study, the application of metabolite and bacteria to check the impact on growth of sunflower in saline-field and pot conditions showed that synergism of 2% EPS and bacteria was the most effective treatment in promoting growth and protecting plant. For only EPS treatments, EPS extracted from PE3 was selected (in accordance with the objective of the study), however, for bacteria plus metabolite treatment, respective EPS was used to ensure compatibility. Due to excellent PGP and salt- tolerance properties exhibited by AF7, the bacteria was selected and complete characterization of AF7-EPS is ongoing. The selection of 2% EPS for the combination treatment was done to ensure the cost-effectiveness of bioformulation. Elucidating the

role of sole bacterial inoculation, the production of phytohormones, chelation of nutrients by PE3 and AF7 along with salt-tolerance ability could have possibly induced growth promotion in plants. PGPB alter the root structure through IAA production, which thereby restrict uptake of sodium ions and enhance the chelation of nutrient and water molecules under saline conditions (Egamberdieva et al. 2019). Another phytohormone GA, along with enhancing germination rate also increases antioxidant activity, chlorophyll synthesis, root-shoot length, plant biomass and inhibits lipid peroxidation under salt stress (Maggio et al. 2010). The uptake of nutrients including P, Zn, K and Fe has also been linked to salt-stress mitigation in plants. These nutrients are involved in regulating various biochemical processes, maintaining osmotic balance, reducing uptake of sodium ions, increasing antioxidant activity, maintaining the integrity of membrane and regulating stomatal conductance (Bargaz et al. 2018; Hasanuzzaman et al. 2018; Mozafari et al. 2018). Both the selected isolates (PE3 and AF7) with multiple PGP traits and with support of EPS were not only able to subside the toxicity of salts in plants but also enhance the growth and yield of sunflower in saline conditions. Tahir et al. (2019) observed positive correlation between auxin production, K^{+}, Ca^{+2} uptake, Na^{+1} exclusion by *Bacillus sp*. and plant biomass, tuber weight of potato under salt stress. Han et al. (2021) reported that the potential of *Burkholderia pyrrocinia* strain P10 to produce IAA, siderophores, solubilize P and produce ACCD upto 5% NaCl and 20% polyethylene glycol, significantly elevated the growth of peanut under saline conditions (100- and 170-mmol/L NaCl).

Stress resilience in plants inoculated with PE3 and AF7 could be possibly due to the tendency of isolates to accumulate osmolytes, presence of antioxidant systems and reduction potential. Yang et al. (2009) showed that the presence of these ST traits elicite "Induced Systemic Tolerance" in plants. Interestingly, Donati et al. (2013) reported that

IAA induces EPS production and biofilm formation. Hence, the mutualistic relationship between the metabolite and bacteria could have acted synergistically with multiphasic roles enhancing the growth parameters of plants under salinity stress. Although *P. entomophila* has been popularly used as entomopathogenic bacteria but there are very few reports available regarding use of this bacterium as PGPB under saline soil conditions. Also role and ability of PGPB strains of *Alcaligenes* in saline environment is not much known and there are few reports defining their role in salinity mitigation. As observed from the pot and field study, application of bacteria alone (without EPS) was lesser impactful in comparison to combination. This shows the role of EPS in microbial survival as well as plant growth promotion.

Roots are the typical site of mineral and water exchange and also the hotspot for plant-microbe association (Jacoby et al. 2017). Apart from direct improvement of root structure through IAA production, EPS forms a rhizo-sheath safeguarding the microbe-plant interactions. These rhizo-sheaths are referred as 'biofilms' inside which microbes multiply, insulated from external stresses, microbial competitions and thrive in the nutrient and water enriched micro-environment (Saleh-Lakha and Glick 2006). EPS are 97% hydrated molecule (Sutherland 2001) and these macromolecules are the basic constituents of biofilm formation (Boukhelata et al. 2019). This suggests that EPS could have protected the microbes prior to onset of salinity symptoms and thus the bacteria were able to survive for longer time without losing any of their beneficial abilities. Getahun et al. (2020) also confirmed that the selection of bacteria on the basis of their EPS production and biofilm formation is very crucial for land remediation especially in saline and water-stressed environment. In the same study, it was observed that the strains of *P. fluorescence*, *Pseudomonas polymyxa* and *P. putida* drastically increased the biofilm formation and EPS production in presence of 100 and 150 mM NaCl and

the ability was increasing with salt-concentration. EPS and biofilm safeguarded the viability of bacterial cells against hyperosmotic surrounding in the rhizosphere and thereby improved growth of acacia plant under salt and water stress. Ansari et al. (2019b) reported that inoculation of EPS, IAA, ACC deaminase producing and P solubilizing *B. pumilus* strain FAB10 to wheat under salt-stress elevated the biofilm formation on the root surface and protected the plant from stressed surrounding.

The current study also proved the concept by showing that the addition of EPS to PE3 and AF7-bioformulation not only enhanced the root colonization but also helped in maintaining the population (of inoculated PGPR) even under salinity stress conditions. Tewari and Sharma (2020) also reported *Rhizobium* bioformulation supplemented with EPS increased the rhizobia population in saline lands and improved the growth of pigeon pea due to incremented nodulation and rhizobial colonization as compared to sole bacteria application and untreated control. The combined treatment of maize seed with bacteria along with EPS enhanced the plant biomass, root/ shoot length, improved soil water content and also stimulated protein and sugar content of osmotically stressed plants (Naseem and Bano 2014). Kasim et al. (2016) reported that biofilm formation by *B. amyloliquefaciens* enhanced the tolerance and survival rate of barley seedlings under salinity stress. Kumar et al. (2020) reported that composition of EPS changes under salinity and drought stress and the biopolymer is produced by soil microbes in response to the onset of stresses. EPS are produced as slime by bacteria and the polymer binds to soil through Van der Waals forces, hydrogen bonding, cation bridges, and anion adsorption mechanisms. Therefore, when EPS producing microbes are inoculated, plants display resistance and tolerance against abiotic stresses such as salinity and drought. The present study also highlighted that EPS is an essential plant growth regulator. The positive correlation between carbohydrate, protein, phenolic contents,

along with salt-tolerance traits in plants and applied EPS concentration (from correlograms), suggest that EPS is linked to their accumulation (in plants). However, deeper molecular-insights are required to determine the correlation between EPS production and other PGP and stress mitigating traits.

Being an efficient sodium ion accumulator (as observed from flame photometry result, FT-IR and XRD analysis), EPS may have reduced the availability of sodium ions to the plants extending their survival and growth rate. Ashraf et al. (2004) found that EPS producing *Bacillus* sp. restricted the uptake of sodium ions in wheat seedlings due to their chelation strategy. Although sodium levels in plants were not checked in the present study, yet results directly established the potential of EPS (of PE3) to chelate sodium ions (flame-photometry, XRD and FT-IR results). ROS leads to chlorophyll degradation (Abd El-Ghany and Attia 2020), however, due to the enhanced antioxidant, hydroxyl scavenging activity and reduction potential in treated plants possibly the chlorophyll content simultaneously increased. Accumulation of flavonoids, carotenoids also leads to the higher fold scavenging of ROS in treated plants. Accumulation of proline has been reported as a major adaption strategy of plants both under stressed and non-stressed conditions, mediating the osmotic balance, preventing cellular damage and scavenging ROS (Gururani et al. 2012). Van Oosten et al. (2018) interestingly related productivity and accumulation of proline with EPS. Therefore, another salt-tolerance response is correlated with EPS. Recently, Sun et al. (2020) reported that EPS producing endophyte *P. alhagi* NX-11 upon inoculation to rice, suppressed salt-induced damage by increasing proline levels, chlorophyll content and K^+/Na^+ ratio in plants. Ilyas et al. (2020) found that EPS and osmolyte producing strain of *B. subtilis* and *A. brasilense* upon inoculation to wheat grown in arid regions of Pakistan, enhanced the germination rate, seedling vigor index, root and shoot length, chlorophyll and

carotenoid content in plants. Also the treated plants showed elevated osmolytes such as proline, sugars, proteins and antioxidant activity. The composition of EPS in the study was similar to that of PE3-EPS *i.e.*, sugars, proteins and uronic acids. Wang et al. (2018) predicted that inoculation of pepper seedlings with salt-tolerant *Bacillus* sp. significantly increased ($P < 0.05$) fresh and dry weight, root length, chlorophyll content, proline accumulation and antioxidant activity in plants.

Conclusion drawn from the properties of EPS highlight that in addition to plant growth improvement, this biopolymer could be a beneficial target to remediate saline soils. Arora et al. (2010) reported improved soil structure by EPS through formation of micro and macro aggregates and thereby elevating water holding capacity of soil. Boukhelata et al. (2019) reported that EPS produced by *Alcaligenes latus* can absorb 1000 times more water than its dry weight. Vardharajula and Ali (2015) also reported that EPS produced by *P. putida* GAPP45 caused about 50 % soil aggregate stability under salt, drought and temperature stresses. The presence of glucuronic acid and high sugar content in EPS was reported as the main reason for improved aggregation; similar composition was also found in PE3 extracted EPS. Cania et al. (2019) highlighted an interesting fact through long term field experiments, stating that micro-aggregates glued by EPS and LPS are more stable than macroaggregates involving root binding. Similarly, inoculation of *Paenibacillus* KLBB0001- a strong EPS producer (isolated from Gurbantünggüt Desert) in desertic soil to recover biological soil crusts (BSCs) after one year of treatment stimulated heterogenic microbial community, increased bacterial population, and nitrogen and phosphorous content of soil. The treatment also showed that gluing-polymers (EPS) connected sand grains and reduced soil water evaporation (Wu et al. 2014)

This study highlights some novel and interesting concepts regarding responses of bacteria to salt stress and the involvement of metabolites in mitigating the stress. Optimizing the potential of isolates, PGP and stress resilience traits were contrasted against salinity to ensure their action in saline fields. The properties were statistically analyzed and the dominant responses were characterized to mimic the consecutive actions of bacteria when exposed to salinity (in lab and field conditions). To ensure survival, bacteria exponentially increased EPS production and even modified the properties of the polymer. For better understanding, EPS was applied to sunflower plants (alone and in combination with bacteria) grown in saline fields as well as in pots with saline soil. Significant increase in plant growth highlighted that EPS had direct role in inducing stress resilience in plants along with the importance of its components in promoting plant growth was established. Thus, this study can be a breakthrough to promote the yield of plants and also to reclaim the barren saline lands in a cost-effective, non-polluting and sustainable manner.

CONCLUSION

Salinity is a major issue declining the productivity and quality of agricultural ecosystems. Expanding population has further stressed the food security putting constraint to achieve the target of 'zero hunger'. Focusing on the ecofriendly approaches, microbial technology involving PGPR can be the best solution to mitigate salt stress and promote growth of plants. In the present study salt-tolerant bacteria were isolated from highly saline areas of Kanpur Dehat of Central Uttar Pradesh (EC 10.5-19.5 dS/ m). Apart from salt-tolerance the strains were also categorized as effective plant growth promoters even in the presence of high salt concentration. Elucidating the mechanisms involved in salt-tolerance, EPS production was found to be the most dominant response of bacteria along with other salt-tolerance traits. EPS was further characterized to establish the role in salt stress and the changes induced in its properties under saline stress. The study further proved the potential of EPS and the isolated strains through field and pot studies taking sunflower as test crop. Contrasting with the untreated control, the treated plants were found to be healthier and stress resistant when EPS and bacteria were applied in combination. This work through different characterization techniques and statistical analysis directly established the significant role of EPS in mitigating salt-stress in plants as well as microbes. Therefore, this study is a novel approach to reclaim saline soils through microbial techniques and popularize tailor-made bioformulations using beneficial microbes as well as their metabolites.

SUMMARY

Soil salinity is one of the most detrimental abiotic stress limiting the growth and yield of plants. Salt-stress induces various negative responses in plants at all developmental stages such as germination, seedling development and maturation and vegetative and reproductive growth. Oilseeds are important rotational and alternative crops for marginal fields and can be utilized simultaneously for food, fuel, fodder and fiber. However, the increasing salinization of lands due to climate change and other ill-agricultural practices, reduces the oil content and impairs the growth parameters mainly due to osmotic and water stresses. Sunflower, an important oilseed crop with various health benefits, has also seen significant reduction in yield and cultivation area due to salinity. Hence, there is an emergent need to adopt biological, cost-effective and potent sustainable approach to mitigate salt stress in crops and remediate the saline fields for better yield. Microbe-based inoculants designed using potent salt-tolerant PGP bacterial strains and their associated metabolites could be way-forward to ameliorate saline marginal fields and promote the growth of plants under all environmental conditions.

In the present study, salt-tolerant bacteria were isolated from saline soil of Kanpur Dehat region of Central Uttar Pradesh (characterized as highly saline; EC 10.5-19.5 dS/ m) and were further screened on the basis of salt tolerance level and PGP traits. In total 42 bacteria were isolated from highly saline soil, of which 12 were selected on the basis of salt-tolerance and were further tested for PGP traits and fluorescence under UV light. The phenotypic characterization of isolates confirmed that all colonies were pigmented and were mostly mucilaginous with smooth margins (except T1K1). The isolates were rod shaped and 90% were Gram negative with fast growth rate (generation time 2-4.5 h). About 81% of bacterial isolates showed fluorescence under UV light. The isolates were further refined on the basis of their ability to tolerate 8% NaCl. It was observed that bacteria were able to grow in the pH range 6-10 and temperature between 15-35ºC,

however, acidic conditions and very low and high temperatures (5ºC and 45ºC) inhibited the growth of most of the isolates. Regarding utilization of various carbon and nitrogen sources by isolates, it was found that glucose was the most preferred carbon source and all the bacteria utilized yeast extract, KNO_3 (except T1K6), $NaNO_3$, tryptophan and NH_4Cl as nitrogen sources. Biochemical characterization of isolates revealed that most of them showed ammonia production (all), amylase production (93%), PHB accumulation (85.71%), growth on GPA (76%) and were positive for VP (79%) and citrate tests (70%). Only few bacteria were positive for protease (19%), cellulase (17%), H_2S production (10%) and MR test (7%).

Isolates PE3 and AF7 were finally selected for future characterization on the basis of their excellent plant growth promoting activities and salt tolerance. Survival and growth rate of isolates under salt stress were estimated through salinity curve. The response of salt stress in both the isolates was checked through salinity curve evaluating their survival and growth rate. Growth of both the isolates was unaffected upto 2% NaCl condition and slightly reduced at 4% NaCl. However, further increase in salinity significantly reduced the growth rate. Mean doubling time of 270 min and 240 min was observed for PE3 and AF7 respectively, under non-saline conditions, which increased to 275 min and 246 min (respectively) at 2% NaCl. The doubling time was maximum at 8% NaCl i.e., 743 min and 613 min for PE3 and AF7 respectively.

All the bacteria were able to produce EPS and 30, 35, 35, 6, 29, 31 and 29 were positive for zinc solubilization, siderophore production, GA production, IAA production, phosphate solubilization and HCN production respectively. The quantitative analysis of the PGP properties revealed that PE3 and AF7 showed best plant growth promoting attributes and thus were further characterized against the gradients of salinity. Phosphate solubilization by PE3 and AF7 was found to be maximum at 2% NaCl,

increasing from non-saline conditions. However, PE3 was a better phosphate solubilizer and the property was maintained upto 6% NaCl while for AF7 no chelation was found above 2% NaCl (coefficient of determination R^2 = 0.818, correlation coefficient r = -0.905 for PE3; R^2 = 0.799 and r = -0.846 for AF7). Similar trend was noted for zinc solubilization which increased from non-saline conditions to maximum at 2% NaCl and then declined. There was negative correlation found between zinc solubilization and salinity (r = -0.820 for PE3 and r = -0.790 for AF7) and the regression analysis established the model of negative relationship. Only PE3 was able to solubilize K, which was maximum at 2% NaCl and maintained upto 4% NaCl, but no solubilization was found at 6% and 8% NaCl. Again negative correlation with good fit regression model was established between salinity and K solubilization in PE3 (R^2 = 0.815, r = -0.905). Both the strains AF7 and PE3 showed siderophore production upto 6% NaCl conditions and it was maximum at 2% NaCl. Salinity imposed negative impact on the production beyond 2% salt level and R^2 value of 0.599 (for PE3) and 0.921 (for AF7) supported the model. Phytohormone production (IAA and GA) by both AF7 and PE3 was maintained even upto 8% NaCl, while the maximum production was reported at 2% NaCl. The linear regression analysis and negative correlation coefficient established reverse relationship between salinity and phytohormone production. The strains were capable of producing HCN even under saline conditions and showed biocontrol activity against *M. phaseolina* (55% inhibition by PE3 and 48% by AF7), *A. brassicae* (63% inhibition by PE3), *F. oxysporum* (60% inhibition by PE3 and 51% inhibition by AF7). However, among all the isolated bacteria (42) T1K1 was the most potent biocontrol agent against diverse spectrum of fungal phytopathogens such as *M. phaseolina, F. solani, F. oxysporum* and *A. brassicae* inhibiting all of them by more than 50%. Exposure to salts led to 132% increase in EPS production (at 2% NaCl) for

PE3, comparatively AF7 was better EPS producer and there was exponential increase by 263% at 2% NaCl (as compared to non-saline conditions). Though both AF7 and PE3 were able to produce EPS upto 8% NaCl but production was highly reduced above 4% NaCl and the statistical analysis was unable to establish a clear relationship between salinity and EPS production. Both the isolates (PE3 and AF7) were also found to exhibit ACC deaminase activity which is an essential trait to mitigate salt stress in plants by reducing the levels of 'stress ethylene'.

Apart from PGP properties both AF7 and PE3 also showed significant salt-tolerance responses upon exposure to salinity. It was found that except reducing power all the other properties of bacteria (both PE3 and AF7) were positively correlated with salinity ensuring the survival of bacteria. The selected isolates (PE3 and AF7) were capable of accumulating sodium ions and simultaneously synthesizing osmoprotectant proline to regulate cellular equilibrium and osmotic tolerance. Further, reduction potential, antioxidant activity and hydroxyl scavenging activity shown by PE3 and AF7, even under saline conditions confirmed the scavenging of ROS, which are generated under salt stress leading to oxidative damages. The strains PE3 and AF7 were capable of biofilm formation and the activity was increasing with salinity. Biofilm formation by PE3 and AF7 was maximum at 4% NaCl, regression analysis reported poor-fit model for salinity and biofilm formation. Biofilming of PE3 was checked through SEM analysis and confocal microscopy. Analyzing the biofilming activity on glass surface under various saline conditions, it was found that the density of biofilm was increasing with salinity with maximum at 4% NaCl and almost negligible at 8% NaCl. Macro-colonies encased in EPS matrix were seen in bacteria exposed to salts (except at 8% NaCl).

Biofilming on root surface as observed through SEM showed that in comparison to control untreated roots, bacteria (PE3) and crystals of sugars (of EPS) were visible sticking to the root surface. Comparing the biofilming and root colonization upon addition of EPS to bacteria, it was found that the population density of bacteria was increased and macro-colonies trapped in mucilaginous matrix were seen. Biofilms on root surface were also observed through confocal microscopy and rod shaped green fluorescent bacteria in an encapsulated microenvironment were seen. Bacterial cells calculated through *BioFilm Analyzer* software revealed significant increase when EPS was added to bacterial treatment.

Concluding from the PGP and salt-tolerance traits of bacteria against salinity gradients and after statistical analysis it was revealed that PGP properties were dominant upto 2% NaCl and then bacteria triggered the tolerance mechanisms to ensure survival.

The identification of isolates was done by proteomic (MALDI-TOF MS biotyping) and genomic analysis (16S rRNA sequencing). Identification through MALDI-TOF MS analysis confirmed that the isolate PE3 is the member of genus *Pseudomonas*. The 16S rRNA sequencing showed 100% similarity with *Pseudomonas entomophila* L48 (T) (accession number CT573326) through BLASTn and Ez Taxon analysis. AF7 was confirmed as *Alcaligenes faecalis* spp *faecalis* DSM 30030T through MALDI-TOF MS analysis and showed 99% similarity with *Alcaligenes faecalis* strain NBRC 13111 in 16S rRNA sequencing. The other isolates CS5 and SS2 were identified as *Bacillus pumilus* DSM 27T DSM (MALDI-TOF MS analysis), AF2 as *A. faecalis* strain NBRC 13111 (99% match), the nearest homolog of S1C was *Cellulomonas pakistanensis* (92.78% match) through 16S rRNA sequencing. Both PE3 and AF7 were submitted to NAIMCC, India, an international culture-collection center with accession number NAIMCC-B02324 and NAIMCC-B02325 respectively.

The study further characterized the most important metabolite involved in survival of bacteria and plant growth under saline conditions *i.e.,* EPS. EPS of PE3 (confining to the objective of the study, mentioning the application of fluorescent pseuodmonads) was selected and purified EPS extracted at different saline conditions was characterized for change in properties. The compositional analysis (through colorimetric assay) revealed that sugar, protein and phenolic content of EPS was maximum at 2% NaCl and minimum at 8% NaCl. FT-IR analysis further showed the presence of hydroxyl group characteristic of carbohydrate ring (3600-3200 cm^{-1}), CH stretching mainly of methyl or methylene group present in hexoses (3000-2850 cm^{-1}) along with free carboxyl groups, carbonyl groups, amino and peptide content, P=O stretching, glycosidic linkage, alkyl halides and α-D glucan. The major difference reported in EPS at different saline conditions was that O-acetyl ester linkage bond of uronic acid was present only in non-salinized EPS and absent in salt-exposed EPS. The group is significant for chelation of cations (such as Na) and thus prove the binding of salts to EPS under saline conditions.

GC-MS analysis showed that melezitose was the monomeric unit in all PE3-extracted EPS. It is being reported for the first time in any microbial EPS. The percent composition of melezitose was changing with salinity (calculated from peak area) and was maximum in EPS extracted at 2% NaCl and minimum under non-saline conditions. However, apart from melezitose, all EPS (except that at 8% NaCl) exhibited additional sugar guanosine (composed of guanine ring attached to ribose). Additional sugars β-D-glucose and β-D-galactose were present only in 2% salinized EPS confirming the results of colorimetric analysis of carbohydrates. 3-deoxy-D-mannoic acid was found only in 4% and 6% NaCl-EPS. The non-carbohydrate unit of EPS consisted of protein, esters (fatty acids) and carboxylic acid and fatty acid content was increasing along with

salinity. The XRD analysis of EPS showed that EPS was semi-amorphous and the degree of crystallinity was increasing with salinity upto 6% NaCl and then reduced at 8% NaCl. The average size of crystallites calculated were 18.306 nm at non-saline condition, 26.488 nm at 2% NaCl and 35.55 nm and 94.30 nm at 4% and 6% NaCl respectively, which reduced to 16.316 nm at 8% NaCl. The diffractogram pattern of EPS extracted at 2%, 4% and 6% NaCl, when compared to diffractogram of NaCl crystals (JCPDS No. 5-0628) showed presence of NaCl in EPS. Exceptionally, non-salinized EPS and 8% salt exposed EPS showed no presence of salts.

The exposure of salinity also changed the morphology of EPS as detected in SEM analysis. With increase in salinity from non-saline condition to 2% NaCl, the thickness of EPS was increasing with denser and compact structure and sugar crystals were clearly visible. At further increase in salinity (above 4% NaCl) the trapped salt crystals in EPS were seen and the irregular matrix changed to more solid crystallized morphology. SEM-EDS analysis revealed that EPS was composed of elements C, O, Na and Cl. However, according to salinity the weight percent of Na and Cl in EPS was found to be increasing with maximum at 4% NaCl and minimum at 8% NaCl.

The study further reported that EPS extracted from PE3 was a potential reducing agent, antioxidant, sodium accumulator, nutrient chelator, biofilming agent, emulsifier and hydrating molecule. Comparing the properties exhibited by EPS against gradients of salinity it was found that the stress-tolerance properties (aforementioned) were minimum at 8% NaCl and maximum in EPS extracted at 2% NaCl. Observing the changes in properties of EPS under various saline conditions, 2% NaCl-EPS (showing maximum properties) was selected for application to plants both for field and pot studies and the effect was studied.

Elucidating the effect of isolates PE3 and AF7 and their respective EPS on sunflower plants under saline conditions, talc-based bioformulations were prepared under aseptic conditions and were applied to pots and fields for two years. The treatments were designed as: various concentrations of EPS (extracted from PE3) (0.1, 1.0, 2.0, and 5.0%), only PE3/ AF7 and 2% EPS and PE3/ AF7 (combination).

In pot study, the plants treated with bacteria and EPS (in combination) showed best increment in growth parameters and even significant improvement in the physicochemical, biochemical and salt-tolerance traits. The metabolite alongwith bacteria (PE3/ AF7) increased the germination rate of plants by 73% as compared to untreated control. Both PE3 and AF7 were also found to be potent root colonizers at various plant growth stages with maximum population density of 7.623 and 6.653 log 10 CFU/ gram of root at 60 DAS for PE3 and AF7 respectively. However, with addition of EPS the colonization further increased to maximum population density of 8.898 and 8.362 log 10 CFU/ gram of root at 60 DAS for PE3 and AF7 respectively. Analyzing the plant growth parameters, 0.1% EPS treatment was least effective and 2% and 5% EPS showed almost similar effect on growth parameters of sunflower plants. The combination of bacteria and metabolite (2% EPS) was the most effective treatment increasing the root length by 179%, 2-fold increase in shoot length, 35% and 86% increase in fresh and dry weight respectively, as compared to untreated plants. Similarly, the head size, stem girth were also enhanced when EPS and bacteria were applied. The combination also increased yield of sunflower as compared to untreated plants by about 49%.

Similar to growth parameters, combinatorial treatments (bacteria and EPS) also showed maximum increase in physiological and biochemical properties of plants. Relatively, PE3 and EPS increased the chlorophyll, carotenoid, flavonoid, phenolic, protein and

carbohydrate contents by 62%, 75%, 49%, 80%, 74% and 4-fold respectively as compared to untreated plants. *P. entomophila* PE3 was found to be a better plant growth promoter under salt stress than *A. faecalis* AF7. Analyzing salt-tolerance traits of sunflower, it was again found that EPS alongwith bacteria were the best in mitigating salt stress through elevation of osmoprotectant level, reducing power, antioxidant and hydroxyl scavenging activities along with increase in relative water content, membrane stability index and least electrolyte leakage.

In field conditions, both PE3 and AF7 were able to colonize the roots of treated sunflower plants at various growth stages. Addition of EPS to the bioformulation helped in maintaining the population of bacteria in the rhizosphere, protecting against the rhizospheric competition and salt stress. Further, EPS and bacteria significantly enhanced the seed viability and increased the germination rate (81%). Similar to pot study, EPS and bacteria (PE3/ AF7) caused highest increment in growth parameters of sunflower. EPS and PE3 caused 49% and 85% increase in root and shoot length respectively, while EPS and AF7 caused 47% and 85% increase (respectively) as compared to untreated control. Similar trend was reported for plant biomass, stem girth, flower head size, seeds per plant and oil content of plants. The combination treatment specifically of PE3 and EPS was best in comparison to all other treatments.

The biochemical properties of plants showed significant changes when metabolite and bacteria were applied under saline conditions. Considering major biochemical properties of plants, 2% EPS and AF7 and 2% EPS and PE3 combination were best followed by 2% and 5% EPS only and least effective treatment was 0.1% EPS. Salt-tolerance traits of sunflower plants showed significant changes upon treatment as compared to non-treated plants. The osmoprotectant levels were triggered in plants treated with EPS and bacteria corresponding to almost 2.5 fold increase as compared to

untreated plants. Reduction potential increased by about 78% and 79% when EPS was applied alongwith AF7 and PE3 respectively as compared to untreated plants. Similarly, hydroxyl scavenging and antioxidant activities were highest in combinatorial treatments.

Statistically analyzing the pot and field data using heat-map and clustering, it was found that results showed two major clusters. One showing lesser effect on plants (including 0.1% EPS, 1.0 % EPS, bacteria only and control treatment set) and the other cluster with more influential effects (including EPS and PE3/ AF7 combination and 2% and 5% EPS). From the dendrograms it was elucidated that bacteria and EPS treatment was the best in pot study. Further, the clusters indicated that effect of bacteria on plant growth promotion and stress resilience was almost equivalent to effect of 1% EPS on plants.

To elaborate the role of EPS in inducing plant growth and survival, correlogram was constructed to signify the relationship between EPS concentration, its properties and traits of plants upon inoculation. The results showed that all the salt-tolerance properties (except electrolyte leakage) of treated sunflower plants were positively correlated with EPS concentration, and salt-tolerance traits induced in plants due to EPS were inter-related, hinting towards some common signaling and molecular pathways.

The present study reports potent salt-tolerant bacteria and characterized the mechanisms involved in tolerance and plant growth promotion under different saline conditions. The study explains the role of EPS in mitigating salt-tolerance and adaption of metabolite according to increasing salt-levels. Through FTIR, GC-MS, XRD, SEM, EDS analysis and confocal microscopy the structure and properties of EPS under various salt-concentration were reported. Novel bioformulations using metabolite and bacteria were designed and optimized to elaborate their role in mitigating salt-stress in

sunflower plants under both field and pot conditions. The study will be a hallmark in reclamation of saline fields and shrinking the losses faced by farmers due to salinity through a sustainable and cost-effective approach.

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APPENDIX

CZAPEK'S MINERAL SALT

Ingredients	**gms/liter**
Carboxymethyl cellulose	5.0
Ammonium nitrate	1.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	1.0
Yeast Extract	0.5
Glucose	1.0
Agar	17
Distilled water	1000 ml

SIMMON'S CITRATE AGAR

Ingredients	**gms/ liter**
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	20.00
Distilled water	1000 ml

UREA AGAR

Ingredients	**gms/liter**
Peptone	1.0
Sodium chloride	5.0
Potassium monohydrogen (or dihydrogen phosphate) phosphate	2.0
Glucose*	1.0
Phenol red solution**	6.0 ml
Urea (20% aqueous solution)	100 ml
Agar	20.00
Distilled water	1000 ml

*Add glucose and phenol red to the molten base and steam for 1 hour, cool to 50^{o}C.
**Add 100 ml urea (filter standardized solution) to the basal medium.

TRYPTONE WATER

Ingredients	**gms/liter**
Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized $CaCl_2$ was added after autoclaving	1.0 ml
Distilled water	1000 ml

SKIM MILK AGAR

Ingredients	**gms/liter**
Skim milk powder	100
Peptone	5.0
Agar	15.0

STARCH AGAR

Ingredients	**gms/liter**
Starch	20.0
Peptone	5.0
Beef Extract	3.0
Agar	15.0
Distilled water	1000ml

MR-VP BROTH

Ingredients	**gms/liter**
Peptone	7.0
Potassium phosphate	5.0
Dextrose	5.0
Distilled water	1000.00 ml

TWEEN 80 AGAR

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5
Calcium chloride dihydrogen	0.1
Tween 80	10
Agar	20
Distilled water	1000 ml
pH	6

GLUCOSE PEPTONE AGAR

Ingredients	**gms/liter**
Glucose	40
Peptone	5
Agar	20
Distilled water	1000 ml

YEAST EXTRACT GLUCOSE AGAR

Ingredients	**gms/liter**
Glucose	10
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4
Yeast extract	1
Agar	20
Bromothymol blue	25 mg

PEPTONE WATER

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5.0

PIKOVSKAYA'S AGAR

Ingredients	**gms/liter**
Yeast extract	0.5
Dextrose	10.0
Calcium phosphate	5.0
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Magnesium sulphate	0.0001
Ferrous sulphate	0.0001
Agar	20
Distilled water	1000 ml

ALEKSANDROV'S MEDIUM

Ingredients	**gms/liter**
Glucose	5
Magnesium sulphate heptahydrate	0.5
Calcium carbonate	0.1
Ferric chloride	0.006
Tricalcium phosphate	2
Mica	3
Agar	20
Distilled water	1000 ml

DWORKIN AND FOSTER SALTS MEDIUM

Ingredients	**gms/liter**
Potassium dihydrogen phosphate	4
Disodium hydrogen phosphate	6
Magnesium sulphate heptahydrate	0.2
Glucose	2
Gluconic acid	2
Citric acid	2
Trace elements	
Ferrous sulphate heptahydrate	1 mg
Boric acid	10 mg
Manganese sulphate monohydrate	11.19 mg
Zinc sulphate heptahydrate	124.6 mg
Copper sulphate pentahydrate	78.22 mg
Molybdenum trioxide	10 mg
pH	7.2

WATER AGAR

Ingredients	**gms/liter**
Agar	8
Distilled water	1000

STAINS, INDICATORS, AND REAGENTS

a) Gram staining

Crystal violet solution: Dissolved 2.0 gm crystal violet in 20.0 m of 95% ethyl alcohol; Gram's Iodine solution: Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilled water; Safranin: Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

b) Indole production test

Kovac'sreagent: Dissolved the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Then added 25.0 ml of hydrochloric acid to the above preparation. Stored the reagent in the refrigerator.

c) MR-VP test

Methyl red indicator: Dissolved methyl red (0.1 gm) in 500 ml of 95% ethyl alcohol. Added distilled water and filtered the preparation.

VP reagent I: 5.0 gm alpha-naphthol was weighed and dissolved in 95.0 ml of absolute ethyl alcohol; VP reagent II 40% potassium hydroxide (KOH)

d) IAA production

Salkowski reagent : 2 ml 0.5M FeCl3 and 49 ml water and 49 ml 70% perchloric acid.

e) Ammonia production

Nessler'sreagent: Dissolved 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Diluted to 1000 ml by addition of distilled water. Allowed to settle for one week. Stored in tightly stopper brown bottles.

f) HCN production

2% Sodium carbonate solution: Dissolved 2 gm of $NaCO_3$ in 100 ml of distilled water. 0.5% Picric acid solution: Mixed 0.5 gm picric acid in 100 ml of distilled water.

g) Amylase production

Gram's Iodine: 1.0 gm Iodine and 2.9 gm of Potassium iodide was mixed and added water to make total of 300 ml.

h) Cellulase production

Congo red dye (1 mg/1 ml solution) is used for staining.

1M NaCl solution is used for destaining

i) Endogenous accumulation of osmoprotectant proline

Ninhydrin reagent: Prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and then 20 ml of 6M phosphoric acid was added and agitated until dissolved

j) Protein content

Alkaline copper reagent: Prepared by adding 1 ml of 2% sodium-potassium tartarate, 1 ml of 1% $CuSO_4.5H_2O$ to 98 ml of 2% sodium carbonate in 10^{-1} M NaOH

k) Siderophore production

CAS dye 121 mg, was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($FeCl_3.6H_2O$) solution was prepared in 10 mM HCl. Subsequently, the solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 ml distilled water) with continuous stirring and was sterilized before use

PUBLICATIONS

Publications

Research articles:

1. **Fatima T,** Arora NK (2021) *Pseudomonas entomophila* PE3 and its exopolysaccharides as biostimulants for enhancing growth, yield and tolerance responses of sunflower under saline conditions. **Microbiological Research** 244: 126671. DOI: 10.1016/j.micres.2020.126671 (**IF: 5.415**)
2. Mishra I, **Fatima T**, Egamberdieva D, Arora NK (2020) Novel Bioformulations Developed from *Pseudomonas putida* BSP9 and Its Biosurfactant for Growth Promotion of *Brassica juncea* (L.). **Plants** 9: 1349. DOI: 10.3390/plants9101349 **(IF: 3.935)**
3. **Fatima T**, Mishra I, Verma R, Arora NK (2020) Mechanisms of halotolerant plant growth promoting *Alcaligenes* sp. involved in salt tolerance and enhancement of the growth of rice under salinity stress. **3Biotech** 10(8). DOI: 10.1007/s13205-020-02348-5 **(IF: 2.406)**
4. Arora NK, **Fatima T**, Mishra J, Mishra I, et al. (2020) Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils. **Journal of Advanced Research** 26: 69-82**.** DOI: 10.1016/j.jare.2020.07.003 **(IF: 10.479)**
5. Arora NK, **Fatima T**, Mishra I, Verma M et al. (2018) Environmental sustainability: challenges and viable solutions. **Environmental Sustainability** 1(4): 309-340. DOI: 10.1007/s42398-018-00038-w.
6. Arora NK, Pandey P, Egamberdieva D, **Fatima T** (2021) COVID-19 pandemic: aggressive research, vaccination, testing, and environmental sustainability are the way forward. **Environmental Sustainability** 4: 443–445

Book Chapters

1. Arora NK, **Fatima T**, Mishra I, Verma S (2020) Microbe-based Inoculants: Role in Next Green Revolution. In: Shukla V, Kumar N (eds) Environmental Concerns and Sustainable Development. Springer, Singapore, pp. 191-246. DOI: 10.1007/978-981-13-6358-0_9

2. **Fatima T,** Arora NK (2019) Plant growth-promoting rhizospheric microbes for remediation of saline soils. In: Arora NK, Narendra K (eds) Phyto and Rhizo Remediation. Springer, Singapore pp.121-146. Doi: 10.1007/978-981-32-9664-0_5

3. Mishra J, **Fatima T**, Arora NK (2018) Role of secondary metabolites from plant growth-promoting rhizobacteria in combating salinity stress. In: Egamberdieva D, Ahmad P (eds) Plant Microbiome: Stress Response. Springer, Singapore, pp.127-163. Doi: 10.1007/978-981-10-5514-0_6

AWARDS

Awards

1. Received **2nd BEST POSTER PRESENTATION AWARD** on the topic, "**Exopolysaccharide producing salt tolerant fluorescent pseudomonads and their role in salinity stress amelioration"** by **ZOOLOGICAL SOCIETY OF INDIA**, in 2 days National Science Day Celebration and Seminar, held during **27th and 28th February, 2018**, at Babasaheb Bhimrao Ambedkar University, Lucknow, India
2. Received **BEST YOUNG SCIENTIST ORAL PRESENTATION AWARD (3rd Position) on the topic, "Characterization of salt tolerance and plant growth promoting properties of isolate *Alcaligenes faecalis* AF7-NAIMCC-02325 and its role in enhancing rice growth under saline conditions",** by **SOCIETY FOR ENVIRONMENTAL SUSTAINABILITY,** in three days Integrated Workshop on "Publication Ethics and Patenting" and International Conference on "Environmental Sustainability: Innovations, Translational Dimensions and Way Forward" held during **10th & 12th February, 2020** at Babasaheb Bhimrao Ambedkar University, Lucknow, India
3. Received **BEST ORAL PRESENTATION AWARD (2nd POSITION)** on the topic, "**Extraction and characterization of EPS from salt-tolerant *Pseudomonas entomophila* PE3 and it's role in promoting growth of sunflower under salinity stress**", by **SERS, Meerut; BBAU, Lucknow in collaboration with CCSU, Meerut and SARS, Meerut,** in 3 days 5th International Conference on Innovative Approaches in Applied Sciences and Technologies, held during **3rd and 5th December, 2021, at** Babasaheb Bhimrao Ambedkar University, Lucknow, India

Microbiological Research 244 (2021) 126671

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Pseudomonas entomophila PE3 and its exopolysaccharides as biostimulants for enhancing growth, yield and tolerance responses of sunflower under saline conditions

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ARTICLE INFO

Keywords:
Soil salinity
Plant growth promoting bacteria
Exopolysaccharides
Sunflower
Plant growth promotion

ABSTRACT

Evaluation of plant growth promoting bacteria and the associated metabolites under saline conditions can be a potential eco-friendly remediation and productivity enhancement strategy. Salt-tolerant *Pseudomonas entomophila* PE3 was isolated from saline soil and screened for plant growth promoting (PGP) traits. The isolate produced indole acetic acid (IAA), gibberellic acid (GA), exopolysaccharides (EPS) and siderophore along with the potential to solubilize potassium (K), zinc (Zn) and phosphorus (P). Maximum stimulation of PGP attributes was recorded at 2% NaCl concentration. To determine the role of EPS, their composition was analyzed (at different salt concentrations) and comparison was done to determine the changes upon exposure to salinity. EPS was found to be rich in carbohydrates, proteins and phenolic compounds. The extracted EPS were also found to possess salt-tolerance properties including antioxidant, hydroxyl scavenging activity, reducing power, emulsification and flocculation potential, and Na^+ accumulation ability. Interestingly, the salt tolerance properties of EPS were enhanced upon exposure to salinity (2% NaCl). Finally, EPS based bioformulation of isolate PE3 was checked through field assay in saline soil. With promising results on growth promotion and improved salinity tolerance attributes of inoculated sunflower plants, the bioformulation of PE3 amended with EPS can be a breakthrough for remediation of saline-agroecosystems.

1. Introduction

Sunflower (*Helianthus annuus* L.), member of the family Asteracea, is an important oil seed crop and is cultivated in about 72 countries for nutritional and medicinal uses. Among the oilseed crops, sunflower ranks fifth with average annual worldwide oil production of 32–44 million tons (Seiler and Gulya, 2015). Sunflower can grow in variety of soils but thrives best in deep loamy and well-drained soils. Sunflower can tolerate slightly acidic to alkaline conditions with pH 6.0–7.5. Sunflower oil has high nutritional value due to larger proportions of unsaturated fatty acids (oleic, linoleic, linolenic) and lower saturated fatty acids (palmitic and stearic). Due to absence of trans fats it is regarded as superior in quality as compared to other vegetable oils (Premnath et al., 2016). Apart from oil, sunflower seed is used as snack, salad garnish, animal feed and in some bakery goods. Sunflower seeds contain phenolic compounds, flavonoids, vitamins, antioxidants, anti-inflammatory, anti-hypertensive, wound healing properties resulting in popularization of the crop around the globe (Fowler, 2006). In addition to cooking/ food, sunflower oil is also used for production of emulsions and margarine formulations (Salas et al., 2015). The non-edible industrial significance of sunflower oil includes lubricants, biodiesel, vegetable-oil based inks; some of the by-products obtained are hulls, soapstocks, lecithins, tocopherols, waxes and meals (Grompone, 2005).

The continuous change in climatic conditions along with polluted environment has caused various abiotic stresses on crops including sunflower, impacting its quality and productivity. Among abiotic stresses, salinity has impacted the sunflower production in several countries. Sunflower is found to be moderately salt-tolerant and can withstand threshold level (2.5 dS/m) of electrical conductivity (EC), but beyond this 30 % and even more yield losses are reported (Khatoon et al., 2000). The highest impact of NaCl toxicity is found on crop's oil

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https://doi.org/10.1016/j.micres.2020.126671
Received 1 August 2020; Received in revised form 29 November 2020; Accepted 11 December 2020
Available online 16 December 2020

plants

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Article

Novel Bioformulations Developed from *Pseudomonas putida* BSP9 and Its Biosurfactant for Growth Promotion of *Brassica juncea* (L.)

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Received: 11 September 2020; Accepted: 9 October 2020; Published: 12 October 2020

Abstract: In this study, *Pseudomonas putida* BSP9 isolated from rhizosphere of *Brassica juncea* was investigated for its plant growth promoting and biosurfactant producing activities. The isolate showed the ability to produce indole acetic acid, siderophore, phosphate solubilization activity and was an efficient producer of biosurfactant. Purification (of the biosurfactant) by thin layer chromatography (TLC) and further characterization by Fourier transform infrared spectroscopy (FTIR) revealed that biosurfactant produced by the isolate belonged to the glycolipid category, which is largely produced by *Pseudomonas* sp. In addition, liquid chromatography-mass spectroscopy (LC-MS) analysis showed the presence of a mixture of six mono-rhamnolipidic and a di-rhamnolipidic congeners, confirming it as a rhamnolipid biosurfactant. Bioformulations were developed using BSP9 and its biosurfactant to check their impact on promoting plant growth in *B. juncea*. It was noted from the study that bioformulations amended with biosurfactant (singly or in combination with BSP9) resulted in enhancement in the growth parameters of *B. juncea* as compared to untreated control. Maximum increment was achieved by plants inoculated with bioformulation that had BSP9 plus biosurfactant. The study also suggested that growth promotion was significant up to a threshold level of biosurfactant and that further increasing the concentration did not further enhance the growth parameter values of the plant. The study proves that novel bioformulations can be developed by integrating plant growth promoting rhizobacteria (PGPR) and their biosurfactant, and they can be effectively used for increasing agricultural productivity while minimizing our dependence on agrochemicals.

Keywords: *Pseudomonas putida*; PGPR; biosurfactant; bioformulation; glycolipids; rhamnolipids; *Brassica juncea*; sustainable agriculture

1. Introduction

Agricultural productivity is a worldwide concern and there is continuous pressure on this sector to meet the rising food demands of the ever-growing population. A report by the Food and Agricultural Organization (FAO) suggests that the world population will reach nine billion by 2050, and hence global agricultural production must increase by 70% [1]. In the race to enhance crop productivity, humans are adversely impacting the environment by applying high amounts of fertilizers and using intensive agronomic practices. Climate change coupled with natural disasters has further exacerbated the situation [2,3]. Therefore, there is an urgent need to develop sustainable farming methods that

3 Biotech (2020) 10:361
https://doi.org/10.1007/s13205-020-02348-5

ORIGINAL ARTICLE

Mechanisms of halotolerant plant growth promoting *Alcaligenes* sp. involved in salt tolerance and enhancement of the growth of rice under salinity stress

Tahmish Fatima[1] · Isha Mishra[1] · Renu Verma[1] · Naveen Kumar Arora[2]

Received: 17 March 2020 / Accepted: 20 July 2020

Abstract
In the present study halotolerant bacteria were isolated from saline soil (EC ~ 11.9). Based on salt tolerance and plant growth promoting characteristics isolate AF7 was selected for further study. It was identified as *Alcaligenes* sp. on the basis of protein profiling and 16S rRNA sequence homology. Interestingly, AF7 showed diverse PGP characters at different salinity levels. While phosphate solubilization activity was expressed up to 300 mM NaCl, siderophore production was shown up to 700 mM, zinc solubilization up to 1000 mM and indole acetic acid (IAA), gibberellic acid (GA) and exopolysaccharides (EPS) production were depicted till 1400 mM. Correlative and regression analysis suggested positive relation between IAA, GA, EPS, siderophore production and zinc solubilization capability of AF7 and salinity up to 300 mM NaCl. EPS was found to be the most significant response and there was 263% increment in presence of 300 mM NaCl when compared to non-saline control. Analysis also showed that while growth promoting attributes were significant up to a threshold salinity level, further increasing the stress deviates the mechanism towards survival involving proline, antioxidant and hydroxyl scavenging activities. Combination of halotolerant AF7 and EPS showed more than twofold increase in the vegetative growth parameters of rice at ~ 170 mM NaCl (EC 9 dS/m). The study shows the mechanisms/metabolites of the plant growth promoting bacterium (PGPB) AF7 prominently involved during the salinity stress. Study also proves that novel bioformulations can be developed by integrative use of EPS and salt tolerant-PGPB which can be effective for saline soils.

Keywords Salinity stress · Plant growth promoting bacteria · Exopolysaccharides · Rice · *Alcaligenes*

Introduction

Soil salinity is a global menace which has challenged the food security and has affected the agricultural sustainability. Soil salinization is the second leading cause of land degradation and desertification (only after soil erosion) affecting several countries across the globe (Orhan 2016). Currently, almost 1 billion hectares of land on Earth is degraded due to salinity (FAO 2015). Arora et al. (2018) highlighted the direct relationship between anthropogenic activities and increase in saline lands. Saline soils are characterized with electrical conductivity (EC_e) greater than 4 dS/m at 25 °C and excess of accumulated salts of Na^+, Ca^{+2}, Mg^{+2}, Cl^{-1}, SO_4^{-2}, CO_3^{-2} (Shahid et al. 2018). Salinity negatively impacts the physical structure and nutrient status of soil and thereby the growth and productivity of crops. The presence of high salt concentration restricts the uptake of minerals, nutrients, and water (Machado and Serralheiro 2017). Plant physiology is also impacted by salt toxicity resulting in reduced photosynthesis rate, lower germination and growth rate along with impaired yield. The hyperosmotic conditions cause loss of water in plant cell sap, and subsequent ionic imbalance results in reduction of enzyme activities, disrupted membrane structure and cell plasmolysis (Munns and Tester 2008; Rahneshan et al. 2018).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-020-02348-5) contains supplementary material, which is available to authorized users.

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Published online: 30 July 2020

Journal of Advanced Research xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Advanced Research

journal homepage: www.elsevier.com/locate/jare

Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils

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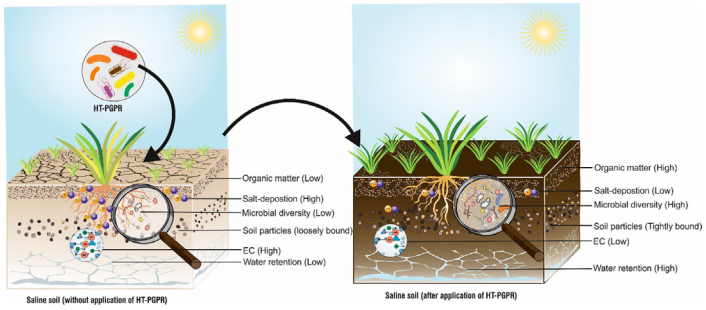
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 May 2020
Revised 26 June 2020
Accepted 7 July 2020
Available online xxxx

Keywords:
Salinity
Plant growth promoting rhizobacteria
Remediation
Sustainable agriculture
Exopolysaccharides

ABSTRACT

The collective impact of climate change and soil salinity is continuously increasing the degraded lands across the globe, bringing agricultural productivity and food security under stress. The high concentration of salts in saline soils impose osmotic, ionic, oxidative and water stress in plants. Halo-tolerant plant growth promoting rhizobacteria (HT-PGPR) are emerging as efficient biological tools to mitigate the toxic effects of high salt concentrations and improve the growth of plants, simultaneously remediating the degraded saline soils. HT-PGPR are involved in alleviating the salinity stress in plants through a number of mechanisms evoking multipronged physiological, biochemical and molecular responses. These include changes in expression of defense-related proteins, exopolysaccharides synthesis, activation of antioxidant machinery, accumulation of osmolytes, maintaining the Na^+ kinetics and improving the levels of phytohormones and nutrient uptake in plants. The modification of signaling by HT-PGPR inoculation under stress conditions elicits induced systemic resistance in plants which further prepares them against salinity stress. The role of microbial-mechanisms in remediating the saline soil through structural and compositional improvements is also important. Development of novel bioinoculants for saline soils based on the concepts presented in the review can be a sustainable approach in improving productivity of affected agro-ecosystems and simultaneously remediating them.

Peer review under responsibility of Cairo University.

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https://doi.org/10.1016/j.jare.2020.07.003
2090-1232/

Please cite this article as: N. Kumar Arora, T. Fatima, J. Mishra et al., Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils, Journal of Advanced Research, https://doi.org/10.1016/j.jare.2020.07.003

Environmental Sustainability
https://doi.org/10.1007/s42398-018-00038-w

REVIEW

Environmental sustainability: challenges and viable solutions

Naveen Kumar Arora[1] · Tahmish Fatima[2] · Isha Mishra[2] · Maya Verma[2] · Jitendra Mishra[3] · Vaibhav Mishra[4]

Received: 3 November 2018 / Revised: 17 November 2018 / Accepted: 21 November 2018

Abstract
Since last century or so anthropogenic activities have intensely metamorphosed the earth's ecosystem and resulted into major environmental changes. Widespread interference of human related activities have resulted in major problems including environmental pollution, land degradation, global warming/climate change, paucity of potable water supply and biodiversity loss. These issues have directly affected the quality and sustainability of the ecosystems. In addition, these activities have resulted in loss of habitats resulting in mass extinction of species which in itself is a matter of great concern. Studies and data clearly show that if present trends continue the conditions are expected to worsen in the coming time and human civilization itself will be in trouble. To minimize this crisis, possible green solutions like use of microbes and biotechnological tools are gaining importance and need further attention in order to lessen or remediate the harmful effects of anthropogenic activities thus ensuring environmental sustainability.

Keywords Environmental sustainability · Anthropogenic activities · Climate change · Greenhouse gases · Pollution · Microorganisms · Bioremediation · Biotechnology · Biofuels · Sustainable agriculture

Introduction

Earth is the only celestial body in the universe where life is known to exist and that too with such a huge diversity. However, it is now becoming more and more inhabitable for most of the species as indicated by steep decline in diversity of flora and fauna. The main reasons behind the worsening conditions for life on earth are the anthropogenic activities. The human interference has resulted in increase in concentration of greenhouse gases (GHGs), climate change, degradation of land, pollution of air water and soil, depletion of non-renewable resources, loss of biodiversity, accumulation of harmful recalcitrant chemicals and several related issues.

Since the last few decades the impact of environmental problems has been highlighted at several forums. In 1960s, the United Nations (UN) discussed the environmental costs of growth-centered or conventional development. In the report 'Our Common Future' (known as the Brundtland Commission), released in 1987 by the World Commission on Environment and Development (WCED), it was stated that development is only 'sustainable' if it 'meets the needs of the present without compromising the ability of future generations to meet their own needs' (WCED 1987). WCED global agenda was an initiative to work on sustaining the planet for better future. Similarly, the UN Conference on the Environment in Stockholm and on Environment and Development (UNCED) in Rio de Janeiro (1992) accepted that the un-sustained human activities towards environment will cause huge, irreversible damage to life on earth (http://www.un.org/geninfo/bp/enviro.html). The UN Agenda 21 was to promote sustainable development undertaken at the Earth Summit held at Rio de Janeiro. The agenda formed the basis for a "global partnership" to encourage cooperation among nations for sustaining life on earth by protecting the environment while simultaneously resulting in growth. For reducing the climate change, countries of the world adopted the Paris

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Published online: 11 December 2018

Environmental Sustainability
https://doi.org/10.1007/s42398-021-00206-5

EDITORIAL

COVID-19 pandemic: aggressive research, vaccination, testing, and environmental sustainability are the way forward

Naveen Kumar Arora[1] · Piyush Pandey[2] · Dilfuza Egamberdieva[3] · Tahmish Fatima[4]

Coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), first reported in December 2019, from Wuhan, China, has been declared as health, social and economic disaster unlike any other since World War II. The irreversible socio-economic changes brought by the pandemic have already disrupted the efforts to achieve the Sustainable Development Goals (SDGs) and even reversed the progress of some goals such as food security, inequality, health and education systems, economy, biodiversity, and environment conservation. Although some species of coronaviruses are well known as human pathogens, for example, severe acute respiratory syndrome coronavirus (SARS-CoV) and middle east respiratory syndrome coronavirus (MERS-CoV), both of which caused outbreaks in very recent past, yet mankind was caught by surprise and under-preparedness after the arrival of the contagion, COVID-19. It is important to research upon what went wrong because of which a novel virus ran through the globe and overwhelmed the health system in almost all the countries. Yes, there are successes seen in vaccine development and health systems, but have we learnt about the root cause of such pandemics related to frequent arrival of zoonotic pathogens in human population?

'Anthropocene epoch' with human population touching 8 billion, and anthropogenic activities causing huge impact on natural ecosystems have resulted in a raging environmental pandemic on the planet since last few decades. This has resulted in several issues including the transmission of novel zoonotic microbes to humans. As per World Health Organization (WHO) about 70% of the novel pathogens arriving in human population are zoonotic in nature. Research has shown that novel coronavirus is a zoonotic virus and most likely was transmitted to humans from wild animals. Bats and pangolins are the most common reservoirs of the coronaviruses. With a loss of around seven million hectares of dense forests each year; one million animal and plant species on the verge of extinction; world heading towards 3.2 ℃ rise in temperature by the end of this century; 75 per cent of the terrestrial and 66 per cent of marine areas being disturbed; the arrival of a zoonotic microbe in human population is becoming ever more likely (Mishra et al. 2021). The breaching of multilayered protection system of natural ecosystems due to deforestation, fragmentation of habitats, biodiversity loss resulting in extinction of natural hosts and climate change are the key factors driving the movement of zoonotic pathogens. Additionally, unplanned urbanization, easy transportation, poorly managed "wet markets" or slaughterhouses, trade in bush meat, warmer environment due to climate change and unorganized livestock cultivation are important factors as well. Global warming resulting in melting of permafrost is a warning sign for arrival of novel pathogens which are dormant since ages (Hofmeister et al. 2021). Apart from the environmental factors there are other challenges associated with a novel virus such as SARS-CoV-2. The development of mutants due to high rate of infection in the population is a challenge. Mutants can be more contagious, dangerous and may result in lower effectiveness of the vaccines. The pandemic is still in a dynamic phase and strategies must be developed accordingly to tackle the ever changing situation due to the virus. We need to be ahead of the evolving virus to control the pandemic as early as possible. In February 2021, International Science Council (ISC) has already established a multidisciplinary Oversight Panel with experts and technical team to report

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Published online: 09 September 2021

Microbe-based Inoculants: Role in Next Green Revolution

9

Naveen Kumar Arora, Tahmish Fatima, Isha Mishra, and Sushma Verma

Abstract

Increasing food demand, with growing population, has been a major concern throughout the globe. The aim can only be achieved with the onset of next green revolution being much defined by sustainable approaches. The past green revolution had its negative impact due to excessive use of agrochemicals contaminating the environment and further challenging the food security. Henceforth, designing the blueprint of next green revolution requires the application of effective and sustainable approaches which enhance the yield of plants while still maintaining the decorum of sustainability. In this regard, microbes have been concluded as the best players finding their roles in plant growth promotion and also stress management. Currently, there are several bacterial-, fungal-based inoculants available in the market along with genetically modified organisms, forming the base of upcoming green revolution. Thus, the future of sustainable agriculture is related to the efficiency and action of these microbes.

Keywords

Microbial inoculants · Green revolution · Environmental sustainability · Plant growth-promoting rhizobacteria (PGPR) · Stress

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V. Shukla, N. Kumar (eds.), *Environmental Concerns and Sustainable Development*,
https://doi.org/10.1007/978-981-13-6358-0_9

Chapter 5
Plant Growth-Promoting Rhizospheric Microbes for Remediation of Saline Soils

Tahmish Fatima and Naveen Kumar Arora

Abstract Salinity is responsible for lowering the quality of soil and reducing the agricultural productivity, continuously increasing the area of marginal lands across the globe. Among the biological solutions, application of rhizobacteria has emerged as the effective strategy for promoting plant growth and enhancing the fertility of saline soil. Various direct and indirect plant growth-promoting attributes including phytohormones, exopolysaccharides, osmolytes, antioxidants, and 1-aminocyclopr opane-1-carboxylate (ACC) deaminase production help to combat the negative impact of salinity on plants. Indirect mechanisms such as synthesis of hydrogen cyanide (HCN), antibiotics and hydrolytic enzymes help in inhibiting the growth of phytopathogens in saline conditions. These beneficial microbes also improve the structure and quality of soil, rejuvenating their productivity index. Henceforth, the identification and selection of halotolerant microbes and studying the mechanisms involved in salt tolerance would provide a better future in designing amelioration strategies for saline soils.

Keywords Salinity · Rhizospheric microbes · PGPR · Rhizoremediation

5.1 Introduction

Soil salinity is a major issue across the globe increasing by the day in irrigated, semiarid, and arid regions. Salinity has degraded almost one billion hectares of land across the globe, siphoning the fertility and productivity of soil leading to substantial loss of crop productivity (FAO 2015). The scenario is worsening in arid and semiarid areas where each day 7.7 square miles of land is lost due to salinization (Technical Report on Soil Degradation 2000). The impact of global climate change,

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N. K. Arora, N. Kumar (eds.), *Phyto and Rhizo Remediation*, Microorganisms for Sustainability 9, https://doi.org/10.1007/978-981-32-9664-0_5

Chapter 6
Role of Secondary Metabolites from Plant Growth-Promoting Rhizobacteria in Combating Salinity Stress

Jitendra Mishra, Tahmish Fatima, and Naveen Kumar Arora

Abstract Increasing salinity and decreasing crop productivity are the two parallel problems, currently faced in agroecosystems across the globe. In the absence of proper remediation of salt stress in soil, the loss of productivity and quality has raised manifold. The saline-tolerant plant growth-promoting rhizobacteria (PGPR) are being realized as alleviators of salt stress. The capability of salt-tolerant PGPR in harsh environmental conditions could be beneficial for plant survival in saline soils. Secondary metabolites produced by salt-tolerant PGPR have also shown clear-cut role in improving plant physiological conditions under osmotic stress. Salt-tolerant PGPR and their metabolites can be the solution for increasing the productivity and remediation of saline soils in an eco-friendly manner. However, research on salt-tolerant PGPR and their metabolites is still in its primary phase and requires more global attention and application.

Keywords Salinity stress • Plant growth promoting rhizobacteria • Secondary metabolites • Biocontrol

6.1 Introduction

At all stages of growth, crops can face different abiotic and biotic stresses (Atkinson and Urwin 2012; Rejeb et al. 2014). At the global level, these stresses have been found to cause substantial loss in crop productivity and quality (Suzuki et al. 2014; Rana et al. 2016). Although, individually, several vital activities of plants are affected either by single or multiple stresses, impact of salinity is much more serious on overall physiology of the plant. Soil salinization is a global problem, and level of salinity is increasing in several parts of the world including the Mediterranean

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D. Egamberdieva, P. Ahmad (eds.), *Plant Microbiome: Stress Response*,
Microorganisms for Sustainability 5, https://doi.org/10.1007/978-981-10-5514-0_6

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