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

SUMMARY OF

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Tahmish Fatima

M.Sc., CSIR-NET, UGC-MANF SRF

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Under the Supervision of

Prof. Naveen Kumar Arora

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025

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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.