SALT-TOLERANT FLUORESCENT PSEUDOMONADS FOR THE BIOFORTIFICATION OF WHEAT UNDER SALINE CONDITION

A Summary Submitted to the
Babasaheb Bhimrao Ambedkar University, Lucknow
in Fulfilment of Requirement for the Award of Degree of

Doctor of Philosophy
in
Environmental Science

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प्रज्ञा शील करुणा
ESTABLISHED 1996

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Enrollment No. -379/12

Under Supervision of
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Summary

In recent years, the global agricultural landscape has been increasingly challenged by the adverse impacts of climate change, particularly in regions prone to soil salinity. Salinity stress, exacerbated by factors such unconventional usage of chemical fertilizers, erratic rainfall patterns, and poor irrigation practices, poses a significant threat to crop productivity and food security worldwide. Among major staple crops, wheat (*Triticum aestivum* L.) particularly vulnerable to salinity stress, which not only reduces yield but also diminishes the nutritional quality of grains. High soil salinity disrupts ion balance, impairs nutrient uptake, and induces oxidative stress, leading to reduced plant growth, yield losses, and nutritional deficiencies.

In response to these challenges, sustainable agricultural practices are increasingly being sought to mitigate the adverse effects of salinity stress on crop production. Microbial inoculants, such as plant growth-promoting bacteria, offer a promising solution by enhancing plant stress tolerance, nutrient acquisition, and overall productivity. Soil samples were collected from the rhizosphere of wheat plants grown in saline-sodic soil in the Kanpur Dehat region of Uttar Pradesh. A total of forty-five bacterial isolates were isolated on King's B medium. The soil samples had high pH (pH > 9.0) and elevated electrical conductivity (EC > 6), indicating alkaline conditions and the combined effects of salinity and sodicity. All isolates were checked for their salt tolerance activity up to 1500 mM NaCl. All the isolates were Gram-negative, motile, and slightly curved rods. Biochemical tests revealed that 64.4% were urease, 80% were oxidase, 84% were catalase positive while only 6 isolates were indole positive, 2 were MR and 6 were VP positive. (Figure) Further, 84% isolates were found to utilize citrate as sole carbon source. Most of the isolates showed oxidative metabolism while 17% showed fermentation activity in O/F test. Additionally, 71% isolates showed ammonia production and 74% showed gelatin liquification. Only 15%

isolates hydrolysed starch, 17% showed casein hydrolysis, 6% produced lipase, 11% were able to grow at 4°C while none of the isolates showed growth at 42°C. During growth experiments at varying salt concentrations, most bacteria were able to grow up to 1500 mM NaCl concentration on agar plates. The five highest salt-tolerant bacteria were further tested for plant growth promotion under varying salt concentrations. Isolate PWR-1 showed luxuriant growth at 200 mM salt concentration, but growth decreased with increasing salt levels. The doubling time of the bacterium varied under different conditions, including under non-saline conditions, the mean doubling time was lowest (235 minutes) with a slight increase (259 minutes) at 200 mM NaCl and highest (1069 minutes) at 1500 mM NaCl concentration.

Further, all isolates were screened qualitatively for plant growth-promoting (PGP) activities. Isolate PWR-1 was selected for quantitative estimation under varying salt concentrations. PWR-1 exhibited higher phosphate solubilization activity in a medium containing 200 mM NaCl (682±5.24 µg/ml) compared to non-saline conditions (679.08±7.45 µg/ml). However, above 200 mM NaCl, phosphate solubilization tended to decrease. Regression analysis revealed a negative correlation between phosphate solubilization by the bacterium and salt stress, with an R2 value of 0.913. The highest level of IAA production occurred in the medium without NaCl (34.50±2.45 µg/ml). At 200 mM NaCl, the IAA concentration slightly reduced to 32.50±2.45 µg/ml. However, as salt concentration increased beyond 200 mM, phytohormone production declined significantly, with the lowest levels observed at 1000 mM NaCl (5.87±1.12 µg/ml). Interestingly, PWR-1 was unable to produce IAA above 1000 mM NaCl. The highest GA levels were observed in the medium without NaCl (55.95±3.96 µg/ml). At 200 mM NaCl, GA production slightly decreased to 53.99±3.25 µg/ml. However, GA production declined significantly as salt concentration increased above 200 mM. The lowest GA levels were observed at 1000 mM NaCl (8.48±1.23 µg/ml). Similar to IAA, PWR-1 did not produce GA above 1000 mM NaCl.

Qualitative analysis revealed that 10% of the isolates showed HCN production, evident from the color change of filter paper (from dark brown to light brown). Isolate PWR-1 was tested

for ACC deaminase production and was found to grow on DF medium plates with ACC and ammonium sulfate as nitrogen sources up to 1000 mM NaCl. The study quantitatively assessed Zn solubilization by PWR-1 using different Zn salts (ZnO, $Zn_3(PO_4)_2$, $ZnCO_3$) across varying NaCl concentrations. Notably, the most substantial halo zone formation occurred with $Zn_3(PO_4)_2$ and highest was without NaCl (4.79±0.070 ZSI). ZnO exhibited optimal solubilization without NaCl (4.64±0.10 ZSI) and gradually decreased with increasing salt concentration. In contrast, $ZnCO_3$ showed the lowest solubilization. All Zn salts exhibited their lowest activity at 800 mM NaCl. Quantitative estimation of Zn solubilization revealed the highest levels of Zn solubilisation was with $Zn_3(PO_4)_2$ and highest Zn solulbilzation was in the medium without NaCl 61.55 mg/l with slight reduction at 200 mM NaCl 61.24 mg/l and was lowest at 800 mM NaCl. ZnO and $ZnCO_3$ also exhibited notable solubilization, with decreasing levels at higher salt concentrations.

Siderophores are molecules produced by bacteria to chelate iron, making it available for their growth. All isolates in the study changed the colour of the medium from blue to orange, indicating siderophore production. Strain PWR-1 exhibited the highest orange zone, prompting further investigation. Quantitative analysis revealed that medium without NaCl showed 94.15 psu siderophore production that was highest followed by 91.45 Psu at 200 mM NaCl. Beyond this range, siderophore production decreased with increasing NaCl concentration. No siderophore production was observed after 1200 mM NaCl. Notably, there was a negative correlation coefficient (-0.9866) between siderophore production and salt concentration, with an R2 value of 0.973. The bacterium PWR-1 produced the highest levels of proline (66.35 µg/ml) at 600 mM NaCl and glycine betaine (59.66 µg/ml) at 800 mM NaCl, with both declining at higher concentrations. DPPH scavenging activity peaked at 800 mM NaCl (91.33 µg/ml) and decreased at higher salt levels. Beyond 1200 mM NaCl, PWR-1 ceased producing proline, glycine betaine, and significant antioxidant activity.

Moreover, PWR-1, which exhibited high plant growth-promoting (PGP) activities, underwent further analysis. MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization

Time-of-Flight Mass Spectrometry) was used for genus-level identification. The closest match was with *Pseudomonas putida*, confirming that PWR-1 belongs to the genus *Pseudomonas*. To delve deeper, molecular identification using 16S rRNA sequencing was performed, in which PWR-1 showed more than 99% homology with *Pseudomonas taiwanensis* DSM21245 strain BCRC17751. The 16S rRNA gene sequences-based phylogenetic tree also confirmed the highest similarity with *P. taiwanensis* DSM21245 strain BCRC17751 (Accession number NR116172.1). Isolate PWR-1 was officially submitted as *P. taiwanensis* to the National Agriculturally Important Microbial Culture Collection (NAIMCC), India, with accession number NAIMCC-B-03362.

PWR-1 was studied for its organic acid production under varying salt stress levels. The medium containing $Zn_3(PO_4)_2$ showed the most significant reduction in pH compared to other Zn salts. Interestingly, this pH reduction displayed a negative correlation with increasing NaCl concentration. HPLC analysis revealed the presence of three organic acids: gluconic acid, with highest production without NaCl (253.35 µg/ml); fumaric acid, highest without NaCl (428.95 µg/ml); and malonic acid, which increased with increasing NaCl concentration, peaking at 800 mM NaCl (428.23 µg/ml) and dropping to 14.96 µg/ml without NaCl. These findings highlight the intricate relationship between Zn salts, NaCl levels, and organic acid production by PWR-1. Further, FT-IR analysis focused on major peaks in the three organic acid fractions also show peaks similar to the organic acid compounds. Additionally, the Zur gene from *P. taiwanensis* strain PWR-1 was successfully amplified using PCR and reference primers. BLASTN analysis revealed 98.71% homology with the Zur gene of *Pseudomonas aeruginosa* strain (WP_015268473.1). The sequence was deposited into the GenBank database under the accession number OR344770.1.

To understand the mechanism of Fe chelation, the siderophore extracted from *P. taiwanensis* PWR-1 using ethyl acetate exhibited positive results. When 0.5 ml of the extract was poured into wells on agar plates and incubated, a distinct and well-defined orange halo zone formed around each well. This clearly indicates the robust iron-chelating capability of the siderophores produced by *P. taiwanensis* PWR-1. TLC was used to separate compounds in the

crude metabolite extract. The crude metabolite of *P. taiwanensis* PWR-1 revealed the presence of various compounds, and a compound (Rf value of 0.70) showed a high affinity for iron, indicative of hydroxamate-type siderophores. The hydroxamate type siderophore was confirmed by spraying FeCl3 solution on the developed TLC plate, forming a brown color. SEM analysis revealed that bacterial cells were prominently present around the surface of plant roots, indicating effective colonization of the plant roots.

Given the promising results of PWR-1, a talc-based bioformulation was developed and tested in both pot and field trials over two years. The formulation showed sustained viability for one year, with a significant enhancement in wheat germination and growth under saline conditions. Pot studies revealed that the application of PWR-1 combined with 50% of the recommended dose of nitrogen (N), phosphorus (P), zinc (Zn), and iron (Fe) fertilizers led to the highest seed germination rate (94%) compared to full fertilizer doses or bacterium-alone treatments. This combination also significantly improved shoot and root length, tiller and spike length, and various biochemical attributes such as chlorophyll content, osmoprotectant production, and antioxidant activity. The treatment notably reduced oxidative stress indicators, contributing to better ion homeostasis and nutrient uptake.

In the field trials, the combined application of PWR-1 and reduced chemical fertilizers led to significant improvements in root and shoot length, fresh weight, dry weight, tiller number, spike length, grains per spike, and overall grain yield. Biochemical analyses indicated higher levels of chlorophyll and carotenoids, enhanced production of osmoprotectants, and increased antioxidant activity in treated plants. This translated into reduced oxidative stress, as evidenced by lower malondialdehyde (MDA) levels and improved nutrient uptake and assimilation. Importantly, the nutritional quality of wheat grains from treated plants showed a marked increase in protein, N, P, Zn and Fe content.

To further assess the impact on nutrient biofortification, wheat grains from different treatments were analyzed for iron and zinc localization and concentration. Treatment with PWR-

1 and reduced fertilizers showed the highest iron concentration and zinc biofortification, as validated by staining and quantitative analysis. These results were validated by atomic absorption spectroscopy (AAS), demonstrating the effectiveness of microbial inoculation in enhancing the nutrient content of wheat grains. Composite soil samples collected before sowing and after field harvest were checked for pH, EC, and Na content, organic carbon, K, Zn, total N, and available P. After harvest, the composite soil sample showed a reduction in pH, EC, and Na content. The levels of organic carbon, K, Zn, total N, and available P increased compared to the soil sample before sowing.

Overall, this study underscores the multifaceted plant growth-promoting activities of PWR-1, including phosphate solubilization, IAA and GA production, siderophore secretion, and organic acid production. These mechanisms collectively contribute to enhanced plant growth, stress tolerance, and nutrient uptake under saline conditions.

In conclusion, the *P. taiwanensis* strain PWR-1 holds significant potential for improving wheat growth, yield, and nutrient content under saline conditions. The combined application of PWR-1 with reduced chemical fertilizers demonstrated remarkable improvements in various growth parameters, biochemical attributes, and nutrient uptake, surpassing both full fertilizer doses and bacterium-alone treatments. The elucidation of molecular and biochemical mechanisms underlying PWR-1's beneficial effects provides valuable insights for future research and application in sustainable agriculture. This study highlights the promising role of *P. taiwanensis* PWR-1 as a biofertilizer for mitigating salinity stress, enhancing productivity, and biofortifying wheat, with important implications for sustainable crop production and food security in saline-affected regions. The use of microbial inoculants like PWR-1 offers a sustainable and eco-friendly approach to agriculture, reducing reliance on chemical fertilizers and promoting healthier, more resilient crops.