Evaluation of salt tolerant fluorescent pseudomonads for antibiotics production involved in biocontrol of *Fusarium oxysporum* f.sp. *lycopersici* causing wilt in *Solanum lycopersicum* L.

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Summary

Changes in climate patterns are dramatically influencing some agricultural areas. Coastal, semi-arid, and dry agricultural regions are particularly susceptible to the effects of climate change on soil salinity. Climate change and agriculture are directly related. Climate change has an immediate impact on soil health, biodiversity, crop productivity, and water use. The frequency and intensity of rainfall, temperature changes, and other extreme weather events will all have an impact on agricultural production; overall, climate change will probably have a negative impact on global agriculture. Increased soil salinity can lead to decreased plant development, lower yields, and in extreme situations, crop loss. Salinity inhibits plants' ability to absorb water by decreasing their osmotic potential, which makes it harder for the plant to take in water. Moreover, soil salinization is thought to be a contributing factor in desertification and is closely linked to processes of land degradation such soil erosion and arable land abandonment. Therefore, it is imperative to implement a biological, economical, and effective sustainable technique to reduce salt stress in crops and restore saline fields for increased agricultural output. Using efficient salt-tolerant PGP bacterial strains and their secondary metabolites in formulation of microbe-based inoculants may be a useful tool for improving saline marginal lands and encouraging plant development in a variety of environmental stresses.

In the present study, salt-tolerant bacteria were isolated from rhizospheric soil of tomato growing saline soil of Kanpur Dehat region of Uttar Pradesh (was characterized as highly saline soil with EC > 18 dS/m and pH 8.8) and were further screened based on salt tolerance level and antagonistic activities against *F. oxysporum* f.sp. *lycopersici*. In total 46 bacteria were isolated on King's B medium (supplemented with 2% salt) from highly saline soil, of which 9 were selected based on salt-tolerance at 8% NaCl

concentration and were further tested for biocontrol of *F. oxysporum* f.sp. *lycopersici* under salt stress. The phenotypic characterization of isolates confirmed that all colonies were pigmented and were mostly mucilaginous with smooth margins. About, 90% were Gram negative and rod-shaped, 95% of bacterial isolates showed fluorescence under UV light. Results from the biochemical tests revealed that 80% of isolates were showing positive results in oxidase test, 64.4% were urease, 80% were oxidase, 84% were catalase positive. The isolates were further refined based on their ability to tolerate 8% NaCl. Only 8 isolates were showing positive results in indole production and 5 were positive in amylase test. Also, 10 isolates were MR and 4 were VP positive. Results from PHB production test showed that 62% isolates were showing positive results and 74% were positive for lipase test. It was observed that in case of oxidation fermentation test 15% of bacterial isolates showed fermentation activity while 85% showed oxidation activity. All the bacterial isolates were found to produce ammonia and 87% were positive in citrate utilization. Further, all the isolates were able to grow on GPA and 26% isolates showed gelatin hydrolysis activity. About 56% isolates in urease test, 68% in cellulase test, 36% in protease test were positive. Out of all 21 isolates were showing nitrate reductase activity.

During growth experiments at varying salt concentrations, most bacteria were able to grow up to 8% NaCl concentration on KB agar plates. The nine highest salt-tolerant and antagonistic (against *F. oxysporum* f.sp. *lycopersici*) bacteria were further tested for plant growth promotion under varying salt concentrations. Selected bacterial isolate SPT26 showed fluorishing growth at 3% salt concentration, but growth decreased with increasing salt levels while beyond 8% salt growth was inhibited. The doubling time of the bacterium varied under different salt concentrationa, including under non-saline conditions. Mean doubling time of SPT26 under non-saline (0% NaCl) conditions was

found to be 240 min which was increased to 264 min and 272 min at 1% and 3% NaCl respectively. Significantly, the maximum doubling time for SPT26 of 480 min and 642 min was reported at 5% and 8% NaCl respectively. Results obtained from the biocontrol test conducted against *F. oxysporum* f.sp. *lycopersici* revealed that SPT26 inhibited the fungal growth by 65% while 62% under stress of 5% of salt concentration.

Further, all isolates were screened qualitatively for plant growth-promoting (PGP) activities. Out of the total, 80% isolates were capable to solubilize phosphate, 85% to solubilize zinc and 56% were solubilizing potassium. Nearly, 90% isolates were synthesizing siderophores and 88% isolates were producing IAA. Only, 68% bacterial isolates were positive in GA production and 95% strains were producing HCN, evident from the color change of filter paper (from dark brown to light brown) which is plays important role in biocontrol activity. Isolate SPT26 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 5% NaCl.

The isolate SPT26 was selected for further studies based on salt tolerance and biocontrol activity under saline stress. Moreover, SPT26, which exhibited high plant growth-promoting (PGP) activities, underwent further was identified through MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) and was used for genus-level identification. The closest match was with *Pseudomonas putida*, confirming that SPT26 belongs to the genus *Pseudomonas*. To get accurate information, molecular identification using 16S rRNA sequencing was performed, in which isolate SPT26 showed 99.92% homology with *Pseudomonas hunanensis* strain LV (Accession number is PP152356.1). The 16S rRNA gene sequences-based phylogenetic tree also confirmed the highest similarity with *Pseudomonas hunanensis* strain LV. The *P. hunanensis* SPT26 strain was submitted to the National Agriculturally

Important Microbial Culture Collection (NAIMCC) in India, and the accession number NAIMCC-B-03806 was allotted for the strain. Out of all 8 isolates were identified but the isolate SPT4m showed 99.55% homology with *Pseudomonas plecoglossicida* strain NBRC 103162 (Accession number is OQ608724.1). The isolate SPT26 showed 99.92% homology with *Pseudomonas hunanensis* strain LV (Accession number is PP152356.1). The isolate SPT2m showed 94.48% homology with *Pseudomonas aeruginosa* strain DSM 50071 (Accession number is OL587505.1). The isolate SPU31 showed 99.85% homology with *Proteus mirabilis* strain ATCC 29906 (Accession number is OQ608784.1) (Table 9).

Pseudomonas hunanensis, was firstly reported by Gao et al. (2014), isolated from soil exposed to long-term manganese contamination in Hunan province, China. The isolation of strain from manganese contaminated site revealed the tolerance against heavy metal which showed efficacy of strain in bioremediation of soil. In this study, SPT26 was studied for antibiotic production under saline conditions. Hence, the secondary metabolites were extracted from the isolate SPT26 produced under stress of 5% NaCl and *F. oxysporum* f.sp. *lycopersici*. The extracted crude metabolites were further purified through TLC and development of three distinct spots with Rf value of 0.76, 0.84 and 0.90, suggested presence of antibiotic compounds. After purification of the crude metabolite, three distinct compounds named as A, B and C were obtained and the antagonistic behaviour of those compounds against *F. oxysporum* was observed in saline conditions (5% NaCl). Among these compounds compound A showed the highest inhibition (68%) and compound C (45%) the lowest inhibition of growth of fungal mycelia while compound B inhibited the growth by 52%. Based on the inhibition percentage compound A and B were selected for the further examinations. Additionally, MIC test was performed and 1% of minimum concentration of the compound was estimated for its application in the tomato plants exposed to infection of *F. oxysporum*.

The FT-IR spectrum showed peak at 3298.29 cm^{-1} representing the presence of pyrrole ring. Peaks at 2947.5 cm^{-1} indicates the stretching vibration of –OH group denotes the existence of alcohol, phenol, and carboxylic acid present in the metabolite sample. Presence of peak at 1661.29 indicated the N–H bending denoting the vibration of primary and secondary amide compounds such as acetamide, carboxamides, benzamides and absorbance peak at 1449.29 cm^{-1} attributed to C=O stretching of carboxyl functional group. Peaks at 1112.91 cm^{-1} and 1021.13 cm^{-1} signify presence of C–F group and alkyl halides. In LC-MS results, major peaks in compound A, B and C were observed at specific retention times, with mass-to-charge ratios (m/z) corresponding to ions detected by the mass spectrometer. These peaks included m/z ratio 256.6 and 258.6 in compound A, m/z ratio 197.4 in compound B and m/z ratio 325 in compound C. Compounds A, B and C were analysed by LC-MS, which demonstrated the presence of antifungal compounds, siderophore and antibiotics. The results signified the presence of pyrrolnitrin, 1-hydroxyphenazine and pyochelin in the compound A, B and C respectively which were further utilized for preparation of bioformulation in combination with SPT26.

The effect of SPT26 and its antifungal compounds on growth and yield of tomato under saline conditions was studied by pot and field experiments. The plants in both the experiments were treated with bioformulation prepared with SPT26 and its antifungal compounds in various combinations. In this experiment, a talc-based bioformulation and liquid bioformulation of *P. hunanensis* strain SPT26 and in combination with its antifungal metabolites was processed in the laboratory. The assessment of bacterial vitality showed the decline in colony-forming units (CFU) after time-period of 12 months. The results from pot experiment and field experiment proclaimed that soil quality after treatment of SPT26 was enhanced with increase in bacterial count, WHC, soil nitrogen, available phosphorus along with decrease in pH, EC, sodium and

potassium content in the soil. The germination rate of seeds inoculated by SPT26 with *F. oxysporum* f.sp. *lycopersici* was 82% and SPT26 only was 85% which reported the 3.5% of increase in germination. In our study SPT26 showed various PGP attributes along with production of osmoprotectants which enabled the plants to survive under stressful environments.

Pot studies revealed that the application of SPT26 combined with pyrrolnitrin and i-hydroxyphenazine led to the highest seed germination rate (96%) compared to plants treated with *F. oxysporum* f.sp. *lycopersici* and uninoculated control group of plants. Additionally, this combination greatly increased the length of the tiller and spike, the shoot and root length, and several biochemical characteristics like the concentration of chlorophyll, the synthesis of osmoprotectants, and the antioxidant activity. Better ion balance and nutrition absorption were facilitated by the notable reduction in oxidative stress markers on application of bioinoculants.

In the field trials, the combined application of SPT26 and combination of SPT26 with pyrrolnitrin and 1-hydroxyphenazine led to significant improvements in root and shoot length, fresh weight, dry weight, leaf area, number of branches, and average number of fruits per plant representing overall plant yield. Biochemical analyses indicated higher levels of chlorophyll and carotenoids, enhanced production of osmoprotectants (proline, flavonoids, carbohydrate), increased protein content and antioxidant activity in treated plants.

Analyzing salt-tolerance traits in tomato leaves, it was again found that the application of SPT26 combined with pyrrolnitrin and 1-hydroxyphenazine 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. Importantly, various plant components such as, saponins, tannins, glycosides, coumarins and alkaloids

content were analysed qualitatively and showed that application of bioinoculation of SPT26 in combination pyrrolnitrin and 1-hydroxyphenazine exhibited the better composition when compared to control plants. Further, nutritional contents in the tomato grown with different treatment was analysed and results revealed that again treatment of SPT26 with antifungal compounds in all combinations were having higher beta- carotene, ascorbic acid and lycopene content in the tomato fruits.

In conclusion, the *P. hunanensis* strain SPT26 was found efficient for improving tomatot growth, yield, and nutrient content under saline conditions in presence of *F. oxysporum* f.sp. *lycopersici*. The combined application bioinoculation of SPT26 in combination pyrrolnitrin and 1-hydroxyphenazine noticeably improved various growth parameters, biochemical attributes, and nutrient uptake, surpassing and bacterium-alone treatments. The elucidation of molecular and biochemical mechanisms of SPT26 could prove beneficial effects providing valuable insights for future research and application in sustainable agriculture. This study highlights the promising role of *P. hunanensis* strain SPT26 as a biocontrol agent under saline stress for mitigating salinity stress along with suppressing the growth of *F. oxysporum* f.sp. *lycopersici*, enhancing productivity, and nutritional value of tomato, with important implications for sustainable crop production and food security in salt- affected agroecosystems. The use of novel microbial inoculants using SPT26, and antifungal metabolites will be a hallmark in reclamation of saline fields and shrinking the losses faced by farmers due to fungal phytopathogens. Offering an environmentally safe and sustainable method of farming, decreasing the need for artificial fertilizers, and encouraging stronger, healthier plants.