Diversity of bacterial endophytes from *Brassica juncea* L. and their plant growth promoting attributes under saline conditions

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Exponential rise in the global population combined with expansion of marginal and stressed land puts enormous strain on the agriculture sector. High yielding crops that are resistant to the debilitating effects of abiotic stress are needed. Salinity is one of the major abiotic stresses that restrict the plant growth at every stage of development and causes osmotic imbalances, disturbances in water homeostasis, ion toxicity and produce reactive oxygen species ultimately causing reduction in crop yield. Traditionally used *B. juncea* L., is an important oilseed crop with various health benefits, has also seen significant reduction in yield and cultivation area due to salinity. Application of microbe-based inoculants like plant growth promoting bacteria provide the sustainable, ecofriendly and cost-effective approach for enhancement of crop productivity. Endophytic bacteria can serve as a good option for the same but very limited literature is available on them for salinity amelioration when compared with plant growth promoting rhizobacteria and endophytic fungi. Endophytes being in close proximity to tissue may provide immediate and direct response to stress causing factors.

In this study, salt-tolerant endophytic bacteria were isolated from root stem and leaf of surface sterilized *B. juncea* growing in saline soil of Kanpur Dehat, Uttar Pradesh (soil characterized as highly saline; EC 10.5 dS/m) and were further screened on the basis of salt tolerance level and PGP traits. In total 68 bacterial isolates were obtained from highly saline soil. The phenotypic characterization of isolates confirmed that 42 isolates were mucilaginous and 26 showed non-mucilaginous appearance of colonies. 24 out of 68 isolates were non-pigmented and rest showd pigmentation. Out of 68 isolates, 69% of the isolates were Gram negative and rest 31% were Gram positive bacteria. All the isolates

were rod shaped (except 2 isolates SS1 and SK1 were round shaped. Biochemical characterization of isolates revealed that most of them showed urease test (68%), oxidase test (70%), catalase test (86%), citrate test (84%), ammonia production (61%) and gelatinase test (64%). Only few bacteria were positive for protease (17%) and starch hydrolysis (15%) tests.

The isolates were further refined on the basis of their ability to tolerate 6% NaCl and 30 isolates that showed salt tolerance of 6% were selected for qualitative plant growth promoting traits. Among the selected bacterial isolates 18,14, 16, 10, 15, 10 and 8 were positive for phosphate solubilization, zinc solubilization, siderophore production, potassium solubilization, ACC deaminase production, biocontrol against *A. brassicae* and HCN production. On the basis of qualitative PGP assay 10 isolates were further selected for quantitative estimation of PGP traits. Isolates RS19, SS7 and LK7 showed best plant growth promoting attributes in quantitative assay and thus were further characterized against salinity gradients. Survival and growth rate of the selected isolates under salt stress were estimated through salinity curve. Growth pattern of the isolates was observed under varying salinity stress (1-8% NaCl) and growth curve was drawn. All the isolates were able to show growth upto 8% NaCl concentration. Mean doubling time of the isolates under non-saline conditions were found to be 260 min (for RS19), 240 min (for SS7) and 180 min (for LK7) which slightly increased on addition of 2% NaCl to 268 min (for RS19), 246 min (for SS7) and 188 min (for LK7). Maximum doubling time was reported on growth at 8% NaCl of 753 min (for RS19), 662 min (for SS7) and 428 min (for LK7).

Isolates RS19, SS7 and LK7 were selected for quantitative estimation of plant growth promotion under varying salt concentrations (1-8% NaCl). Phosphate solubilization by

RS19, SS7 was found to be maximum at non-saline condition, and for isolate LK7 maximum P solubilization was observed at 1% NaCl concentration. RS19, SS7 and LK7 solubilized P upto 8% salt stress. It was observed for zinc solubilization that RS19 and SS7 were able to chelate zinc up-to 6% NaCl whereas LK7 was able to solubilize zinc up-to 8% NaCl. SS7 was however highest Zn solubilizer at non-saline condition it was less efficient at higher salt concentration (6% NaCl and above) than LK7. Isolates SS7 and LK7 were able to solubilize K up-to 6 % salt stress however RS19 was able to chelate potassium from insoluble source (mica) not beyond 4 % salt stress. Regarding the impact of salinity on K solubilization, the chelation activity was maximum at non-saline conditions for all the three isolates. In terms of potassium (K) solubilization, the bacteria also exhibited reduced solubilization under increased salt stress. Siderophore production revealed that maximum siderophore production by SS7 and LK7 was at 1% NaCl and then the siderophore producing ability decreased upto 8% NaCl. RS19 did not show siderophore production at 8% NaCl. LK7 was more efficient siderophore producer as compared to SS7 at all the salinity levels. For phytohormone production SS7 isolate was a potent IAA producer at non-saline condition and the strain was able to produce the phytohormone even at 8% NaCl. However, with salinity the ability decreased and at 8% SS7 its solubilization decreased to 88% of its non-saline IAA production. On the other hand, LK7 produced low concentration of IAA than SS7 at non-saline condition but at 8% NaCl stress production of IAA by LK7 reduced only to 69% of its non-saline IAA concentration. Comparing the GA production against salinity, it was found that in case of LK7 upon exposure to salinity, (from non-saline to 1% NaCl) there was an exponential increase in the phytohormone production and was able to produce GA even at 8% NaCl. The regression models of all the

isolates showed negative correlation with all the PGP with R^2 values ranging between 0.7568 and 0.9817. All the three isolates showed production of ACC deaminase (qualitative analysis) upto 5% NaCl concentration which is an essential trait to mitigate salt stress in plants by lowering the levels of stress ethylene.

Beside PGP properties isolates were also checked for their salt tolerance activities. All the three selected isolates showed substantial level of salt-tolerance responses upon exposure to salinity. Isolates were able to accumulate sodium ions within their cells upto concentration of 33472 to 36543 µg/gm of cell dry weight. All the isolates showed increase in sodium accumulation upto 6% salt concentration and thereafter value the decreased. Similarly for concentration of osmoprotectant proline, hydroxyl scavenging activity and antioxidant activity of the isolates enhanced with increase in salt concentration upto 6% NaCl concentration. However isolate LK7 was able to increase its proline production and antioxidant activity even upto 8% salt stress. The ability of the isolates to show salt tolerance property indicates their ability to reduce the levels of oxidative damage causing ROS produced during salt stress.

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 isolates RS19 and SS7 were the member of genus *Pseudomonas*. The 16S rRNA sequencing showed 100% similarity with *Pseudomonas putida* NY5709 (accession number CP045551) through BLASTn analysis. RS19 was submitted (as NKA35) to NCBI-GenBank with accession number ON150842. It is submitted to culture collection center culture collection centre NAIMCC, ICAR-NBAIM, Mau, U.P., India (as NKA35) with accession number NAIMCC-B-03565. SS7 showed

resemblance of 99.93% with *Pseudomonas azotoformans* CLP-10 (accession number MN594835) and the sequence for 16S rRNA gene was submitted to NCBI with accession number OP317300. Strain was submitted at NAIMCC with accession number NAIMCC-B-03563. isolate LK7 showed 99% resemblance with strain *Bacillus stercoris* YBB10 (accession number MN032425). The gene sequence (1200 bp) of LK7 (as NKA36) was submitted to NCBI GenBank with accession number OR540492. *Bacillus stercoris* NKA36 was subsequently submitted to NAIMCC, India with accession number NAIMCC-B-03562.

Biocontrol activity of the three isolates revealed that isolate SS7 and LK7 were able to show antagonistic activity against fungal pathogen *A. brassicae* which causes leaf spot disease in *B. juncea.* Isolate RS19 did not show biocontrol against the pathogen. In vitro analysis of SS7 and LK7 by dual culture and seal plate method revealed that antifungal volatile compounds may be responsible for inhibition of *A. brassicae.* For further analysis purified metabolite extracts of the isolates were checked for the presence of functional groups. Peaks of carboxylic acid, amine salt, alkane, aldehyde, isothiocyanate, alkane, aliphatic and amine functional groups were present in the metabolite sample of SS7. For metabolite sample LK7 peaks showed the presence of functional groups alkanes, aldehyde, alkynes, esters, saturated aliphatics, primary amines, aromatics, alkyl halides and aliphatic amines. The majority of functional groups found in the analysis are reported to be constituents of antifungal compounds. Further characterization of the metabolite was done by GC-MS analysis revealed that the metabolite sample of SS7 contained 17 volatile compounds and LK7 showed presence of 14 VOCs that are reported previously as antimicrobial compounds. Some of the reported antimicrobial compounds detected by

GCMS analysis of metabolite extract of SS7 include 1,2,3-propanetriol, 1-acetate; 2-piperidinone; 1,3-dioxolanes; pyrrolo [1,2-a] pyrazine1,4-dione; hexadecanoic acid; pyrazine1,4-dione; ergotamine and actinomycin. GC-MS analysis of metabolite extract of LK7 revealed presence of reported antimicrobial compounds including 2,5-piperazinedione; 2-methyl hexanoic acid; hexadecanoic acid; cycloalanylleucine; dioxopyrazine and bornyl isovalerate. SEM micrographs revealed that LK7 and SS7-treated the dual culture plates displayed structural abnormality in fungal hyphae compared to untreated fungal hyphae. It is apparent that fungal mycelia collected from the inhibition zone showed breaks, shrinkage, coiling, dehydration, rupture of hyphae and collapsed hyphal structure.

The invitro study for analyzing the effect of *A. brassicae* on *B. juncea* in absence and presence of SS7 and LK7 and their metabolites. It was observed that both the treatments of bacteria treated infected plants were able to show highest plant growth parameters and disease incidences when compared to metabolite treated or only infected plants. Plant height significantly increased in the presence of bacterial isolates and root and shoot length and germination rate was 129% and 118% and 34% higher respectively in presence of SS7 in infected plants compared to only *A. brassicae* infected plants. Disease incidence was also reduced and highest reduction of 63% was observed in LK7 treatment compared to only fungus treatment.

Reinfection test was performed to confirm the reinfection ability of bacterial endophytes in the plant roots, selected isolates RS19, SS7 and LK7 were aseptically inoculated to the host plant by seed priming and were aseptically for 8 days in culture tubes in the plant growth chamber. The ability of bacterial isolate to colonise root tissue was checked using

SEM revealing root reinfection ability of by all the three isolates. For more confirmation roots isolation procedure was repeated from the roots. The obtained bacterium on the plate was identified again using 16S rRNA sequencing to confirm the reinfection process. Results revealed the presence of same identified bacteria in the isolation process confirming reinfection ability of all the three isolates.

Four talc-based bioinoculant were developped using *Pseudomonas putida* RS19*, P. azotoformans* SS7, *Bacillus stercoris* LK7 and their consortium. Consortium was prepared after checking the compatibility between the strains. The assessment was made to observe the colony-forming units (CFU) over one year of bioformulation. It was revealed that viable cell counts decreased over time. Despite the gradual decrease, the bacterial population in the bioinoculant remained compliant with Biofertilizers regulations for the 12 months. Pot analysis showed highest growth, physicochemical, biochemical and salt-tolerance parameters in plants treated with consortium of the three isolates. Consortium treated plant showed highest germination rates in vitro and in vivo conditions. The combination of all bacterial isolate in consortium was the most effective treatment increasing the root length by 76.73%. There was 2 time increase in shoot length, 98% and 141% increase in fresh and dry weight respectively, when compared with untreated plants. Similarly, the number of pods and weight of 1000 seeds were also enhanced when consortium of bacteria was applied.

The consortium treatment showed Similar results for biochemical and salt tolerant properties of plants. chlorophyll and carotenoid contents in leaves of isolate treated plants compared to control treatments and among them consortium treated plants showed highest chlorophyll and carotenoid contents of 62.22% and 70% respectively. Similarly,

consortium increased the flavonoid, phenolic, protein and carbohydrate contents by 39%, 25%, 2-fold and 69% respectively as compared to untreated plants. After consortium *P. azotoformans* was found to be a better plant growth promoter under salt stress followed by *B. stercoris* NKA36 (LK7) and *P. putida* (NKA 35). Analyzing salt-tolerance traits of mustard leaves, it was again found that consortium was the best in mitigating salt stress through elevation of osmoprotectant level, antioxidant and hydroxyl scavenging activities along with increase in relative water content, and showing least electrolyte leakage.

In field conditions, similar to pot study, consortium instigated highest increment in growth parameters of *B. juncea*. Consortium caused 80% germination of seeds against 30% seed germination in untreated seeds. Consortium inoculation resulted in 113 % and 133 % increase in root and shoot length respectively, while second highest root and shoot length was observed in SS7 treated plant causing 87% and 126% increase respectively compared to untreated plants followed by LK7 and RS19. Similar trend was reported for plant biomass, number of pods, weight of 1000 seeds and oil content of plants. The biochemical properties of plants showed significant changes when consortium was applied under saline conditions. Considering major biochemical properties of plants, consortium was best followed by individual isolate treatments of SS7 then LK7 and least effective treatment RS19. Salt-tolerance traits of mustard plants showed significant changes upon treatment as compared to non-treated plants. The proline level was triggered in plants treated with consortium corresponding to 580.77 µg/ gm proline production compared to only 253.98 µg/ gm proline production in untreated plants. Hydroxyl scavenging increased by about 115% consortium was applied as compared to untreated plants. Similarly, antioxidant activity was highest in consortium treatment followed by LK7, SS7 and RS19.

The present study reports potent salt-tolerant endophytic bacteria and characterized the mechanisms involved in tolerance and plant growth promotion under different saline conditions. The study explains the role of isolate individually or in form of consortium in mitigating salt-tolerance. The selected 3 isolates were able to show plant growth promoting attributes even under various salt levels. Biocontrol potential of two of the isolates SS7 and LK7 and antifungal properties of their metabolites were studied through FTIR, GC-MS and SEM analysis. All the isolates were able to colonize root tissues under aseptic condition. Novel bioformulations bacteria and their consortium were designed and checked over time to elaborate their role in mitigating salt-stress in mustard plants under both field and pot conditions. The study will be a hallmark in growth promotion of mustard growing in saline fields and reducing the dependency of farmers on chemical approaches for enhancing productivity.