Evaluation of salt-tolerant endophytic diazotrophs with ACC deaminase activity for enhancing the yield of *Oryza sativa* L. under saline conditions

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

BY

Chanda Bharti

UGC-SRF
(Enrollment No. 1379/18)

SUPERVISOR

Prof. Naveen Kumar Arora

DEPARTMENT OF ENERGY & ENVIRONMENT
SCHOOL OF ENVIRONMENTAL SCIENCES
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A Central University) (NAAC A++ Accredited)
RAEBARELI ROAD, LUCKNOW, UTTAR PRADESH-226025, INDIA

2024

SUMMARY

In recent years, the adverse effects of climate change have negatively impacted global agricultural productivity, especially in areas susceptible to soil salinity. Salt-stress induces various negative responses in plants at all developmental stages such as germination, seedling development and maturation and vegetative and reproductive growth. Among major staple crops, rice is 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. 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 bacterial endophytes, offers a promising solution by enhancing plant stress tolerance, nutrient acquisition, and overall productivity. The plant kingdom is inhabited by a varied range of endophytic bacteria that thrive symptomlessly within plants as an intrinsic element of host metabolism and function, having commensalism or mutualism relationships with plants. The plant kingdom is inhabited by a varied range of endophytic bacteria which thrive symptomlessly within plants as an intrinsic element of host metabolism and function, having commensalism or mutualism relationships with plants. Rice plants were collected from saline-sodic soil in the Kanpur Dehat region of Uttar Pradesh. A total of thirty-two bacterial bacteria were isolated from roots of rice on nutrient agar medium. The soil samples had high 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 1200 mM NaCl. All the isolates were rod shaped and 80% were Gramnegative, while the remaining 20% were Gram-positive bacteria. About 56% isolates were motile while 44% were non-motile.

All the isolates showed catalase production while 18 isolates were positive for oxidase production. Majority of the isolates (values/ 32) were positive for amylase test (13), lactose fermentation (25), growth on GPA (28), VP test (24) and citrate test (19), and 16 isolates were positive for nitrate reductase.

On the basis of salt-tolerance, proteomic analysis of four bacterial endophytes was done for identification of bacteria. Isolate NKA31 was identified as a match with Bacillus subtilis DSM 10T with a score value of 1.891. As a result, the biotyping analysis using MALDI-TOF MS confirmed the identification of NKA31 at the genus level, specifically as belonging to Bacillus. MALDI-TOF MS biotyping of NKA32 revealed that the bacteria best matched with Bacillus subtilis DSM 5611 with a score value of 1.801 therefore confirming only the genus of the isolate.

Further, MALDI-TOF MS biotyping of NKA29 shows that the isolate best matched with Enterobacter asburiae DSM 17506T with a score of 2.241, hence confirming the genus and species of bacteria. MALDI-TOF MS biotyping results for identification of isolate NKA27 revealed that comparing with the Bruker taxonomy database the isolate was best matched with Bacillus pumilus

DSM 1794 1.478 (score less than 2).

Two isolates (NKA31 and NKA32) based on ACCD production, nitrogen fixing ability and salt tolerance were selected for study. 16S rRNA gene sequence analysis showed that isolate NKA31 showed 100% similarity with Bacillus stercoris JCM 30051(Gen bank accession number OR584336) and NKA32 showed 99.92% similarity with Bacillus altitudinis 41KF2b(T) (Gen bank accession number OQ918302). Both these isolates have been submitted to the culture collection centre (NAIMCC, India) under the accession number NAIMCC-B-03561 (for NKA31) NAIMCC-B-03560 (for NKA32).

Survival and growth rate of isolates under salt stress were estimated through growth curve. The response of salt stress in both the isolates was checked through salinity curve evaluating their survival and growth rate in presence of different salt concentration. Growth of both the isolates was unaffected upto 300 mM NaCl but growth rate significantly decreased with salinity levels above 300 mM NaCl. The mean doubling time of NKA31 was 119 min under non-saline

conditions and it rose to 210, 338 and 463 min at 300, 600 and 900 mM NaCl stress, respectively. Similarly, under non-saline conditions the doubling time for NKA32 was 140 min which increased to 195, 240, 374 min at 300, 600 and 900 mM NaCl, respectively.

In the comparative analysis of various plant growth-promoting traits, NKA31 and NKA32 exhibited the highest levels of ACC deaminase and nitrogenase activity, along with an array of the other PGP traits. Consequently, based on these results NKA31 and NKA32 were selected for further detailed characterization.

The phosphate solubilization activity of NKA31 and NKA32 exhibited an increase with rising salinity levels, highest at 600 mM NaCl for NKA31 and at 300 mM for NKA32. NKA31 demonstrated higher phosphate solubilization capabilities, achieving a concentration of 966.2 ± 4.83 µg/ml at 600 mM NaCl, in contrast to NKA32, which reached 1168.9 ± 14.65 µg/ml at 300 mM NaCl. Zinc solubilizing efficiency for both isolates was highest at 300 mM NaCl stress.

Siderophore synthesis on CAS agar plates displayed an orange to yellow colored zone for both NKA31 and NKA32 inoculated plates. Upon quantitative analysis, it was determined that NKA31 exhibited highest siderophore production efficiency (85.97 ± 0.09 psu at 600 mM NaCl) whereas maximum siderophore production by NKA31 was recorded at 300 mM NaCl (63.68 ± 0.83 psu).

The two isolates exhibited varying behaviour in terms of IAA production when exposed to saline and non-saline conditions. NKA31 demonstrated the highest IAA production (73.25 µg/ml) at 600 mM NaCl, representing a 39.63% increase compared to non-saline conditions. On the other hand, NKA32 reached its peak IAA concentration (58.46 µg/ml) at 300 mM NaCl. The ability of NKA31 and NKA32 to secrete gibberellic acid (GA) in response to NaCl stress was assessed using the culture filtrate. Results indicated that NKA31 produced the highest amount of GA3 (17.77 µg/ml) at 600 mM NaCl while NKA32 exhibited higher GA production (6.45 µg/ml) observed at 300 mM NaCl.

In both NKA31 and NKA32 isolates nitrogenase activity was found to be directly proportional to its concentration. The presence of salt stress had an impact on nitrogenase activity in both isolates. NKA31 exhibited the highest nitrogenase activity, reaching 210.05 nmol/ml/h at 600 mM NaCl. Similarly, NKA32 showed an increased nitrogenase activity of 187.01 nmol/ml/h at 600 mM NaCl, representing a 66.81% increase compared to non-saline conditions. Similar to nitrogenase activity, ammonia production by NKA31 and NKA32 was also highest at 600 mM NaCl. The presence of nitrogen fixing gene (*nifH*) under saline and non-saline condition was confirmed by PCR technique. *nifH* was present in both isolates under non-saline condition whereas under saline condition (at 600 mM NaCl) NKA31 showed the presence of *nifH* gene as well as growth on N-free media making it a potent salt-tolerant nitrogen fixing bacteria. The experiment to check the expression of *nifH* in NKA31 under salt concentration is underway.

Both isolates displayed ACCD production ranging from 50.21–246.17 nmol/mg protein/h. Strain NKA31 demonstrated higher ACCD production compared to strain NKA31. NKA31 showed higher ACCD production (246.17 nmol/mg protein/h) at 600

mM NaCl which was more than 3-fold higher than unstressed condition. The ACCD activity by the isolate NKA32 at different salt concentrations was further confirmed by FTIR spectrum analysis. The presence of amines (NH) and ketone (C=O) groups under different salinity stress showed the ability of the isolate to utilize ACC and cleave it into amines and ketones.

Apart from PGP properties, both NKA31 and NKA32 isolates also exhibit salt tolerance mechanism. It was found that all the salt tolerance properties of *B. stercoris* NKA31 and *B. altitudinis* NKA32 were positively correlated with increasing salt stress. The selected isolates (NKA31 and NKA32) were capable of accumulating sodium ions and simultaneously synthesizing osmoprotectant proline and glycine betaine to regulate cellular equilibrium and osmotic tolerance. In NKA31, the accumulation of proline and glycine betaine increased steadily as the concentration of NaCl increased, reaching 295.91 µg/ml and 490.431 µg/ml up to 900 mM NaCl, respectively. Similarly, NKA32 showed an approximately 81% increase in proline and 224.4% glycine betaine at 900 mM NaCl when compared to non-saline control. Both the isolates (NKA31 and NKA32) had capacity to scavenge free radicals, which are generated under stress condition. Along with PGP activities and salt tolerance mechanism shown by NKA31 and NKA32, biofilm formation plays an important role in bacterial attachment to the surface of plants. In the context of the DPPH assay, NKA31 exhibited an inhibition of over 65% on the DPPH radical, with this property also showing an upward trend in response to salinity. Notably, NKA32 achieved a maximum inhibition of 90.61% at 900 mM NaCl. The antioxidant efficacy of NKA31 reached its peak at 600 mM NaCl, demonstrating an inhibition rate of 186.42% against DPPH and maintained (181.26%) up to 900 mM NaCl. Biofilming ability was found to be maximum (2.52 ± 0.002 for NKA31) and (2.13 ± 0.002 for NKA32).at 900 mM NaCl stress.

The analysis of plant growth-promoting (PGP) characteristics and salt tolerance of bacteria in response to varying salinity levels indicated that PGP traits were prevalent up to a concentration of 600 mM NaCl. Beyond this concentration, the isolates activated tolerance mechanisms to help them for survibility.

The endophytic colonization ability of selected isolates (NKA31 and NKA32) within rice root tissues was examined by quantifying number of colonies reisolated from tissue homogenates of treated and untreated plants grown under non-saline and 90 mM NaCl. After re-isolation, obtained isolates were identified through 16S rRNA sequencing to find the inoculated bacteria. SEM analysis shows that both the isolates could colonize in xylem vessel of rice seedlings under saline (few cells observed) as well as non-saline conditions (large number of bacterial cells found in tissues of rice seedlings).

On the basis of ACCD activity, N-fixation ability along with phytohormone production, mineral solubilization, antioxidant production, osmolyte accumulation and ion accumulation, isolate NKA31 and NKA32 were selected for the preparation of bioformulation. A talc-based bioformulation was developed and tested alone and in combination (based on compatibility) in both pot and field trials over two years. The compatibility or synergistic relationship between the two inoculated bacteria is a crucial factor for their proper functioning. The formulation showed sustained viability for six months, with a significant enhancement in rice germination and growth under saline conditions. Pot studies revealed that the application of NKA31 and NKA32 combined with 50% of the recommended dose of nitrogen (N), phosphorus (P), and potassium (K) fertilizers led to the highest growth parameters (approx. 69%) compared to full fertilizer doses or bacteriumalone treatments. This combination also significantly improved biomass, grain yield, straw yield and biochemical attributes such as

chlorophyll content, protein, soluble sugars, phenols and flavonoids when compared to control plants. Osmoprotectant (proline and glycine betaine), antioxidant (SOD and APX) activity, ROS scavenging activity were also enhanced when plants were treated with bacterial bioformulation in combination with reduced dose (50% NPK) of chemical fertilizer. The treatment notably reduced oxidative stress indicators, stress ethylene contributing to better ion homeostasis and nutrient uptake.

In the field trials, the combined application of NKA31 and NKA32 and reduced chemical fertilizers led to significant improvements in root and shoot length, fresh weight, dry weight, tiller number, tiller/plants, panicle/tiller, and 1000 grain. 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) and ethylene levels and improved nutrient uptake and assimilation. Importantly, the nutritional quality of rice grains from treated plants showed a marked increase in protein, N, P, Zn, K and Fe content. The bioformulation also reduced sodium uptake in plant leaves and grains.

Overall, this study underscores the ACCD activity, nitrogen fixation activity and other PGP traits of NKA31 and NKA32, including phosphate solubilization, IAA, GA siderophore production, These isolate also produced osmolytes and produced antioxidants under saline conditions. These mechanisms collectively contribute to enhanced plant growth, stress tolerance, and nutrient uptake under saline conditions.

In conclusion, the *B. stercoris* NKA31 and *B. altitudinis* NKA32 holds significant potential for improving rice growth, yield, and nutrient content under saline conditions. The combined application of NKA31 and NKA32 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 NKA31 and NKA32's beneficial effects provides

valuable insights for future research and application in sustainable agriculture.

This study highlights the promising role of *B. stercoris* NKA31 and *B. altitudinis* NKA32 as a biofertilizer for mitigating salinity stress, enhancing productivity, enhancing salt tolerant properties with important implications for sustainable crop production and food security in saline affected regions.