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Halo-tolerant plant growth-promoting rhizobacterium *Enterobacter* sp. KUSP04 ameliorates salinity tolerance in MTU1010 rice variety

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Salinity is one kind of abiotic stress that hinders crop productivity, especially in coastal regions. The present study focused on the salinity tolerance of a bacterial isolate identified as *Enterobacter* sp. based on *16S rRNA* gene sequencing and its plant-growth-promoting potential in the rice cultivar MTU1010 (a high-yielding, short-duration variety) under salinity stress. The isolate could tolerate sodium chloride up to 900 mM, but increased cell size was observed under the salinity stress. It was sensitive to neomycin and streptomycin at 100 ppm concentration and positive for indole-3-acetic acid, siderophore, and amino-cyclopropane carboxylate deaminase production. Supplementation of sodium chloride (100mM) in the potting soil had a negative effect on seed germination and retarded growth of the rice plant tested. Inoculation of the bacterial isolate recovered the negative effect up to some extent. However, more plant growth promoting effect of the isolate was observed in absence of the salt stress. Detection of lowest level of proline in the inoculated set and survival test indicated the bacterial inoculum was compatible with the plant and stimulated normal growth of the plant. In addition, the inoculation improved the chlorophyll content of the plant tissues and reduced oxidative stress by expressing antioxidant enzymes like ascorbate peroxidase and catalase. All the data suggested that *Enterobacter* sp. KUSP04 could be potentially used as a bio-inoculant in salinized agricultural lands to increase rice productivity.

Keywords : Antioxidant enzymes, *Enterobacter*, plant growth promoting rhizobacteria, Rice, Salinity

INTRODUCTION

Global warming and the resulting climate change had a direct impact on changes in rainfall patterns, the rise of sea level, frequent cyclones, and subsequent increase of salinity in the agricultural fields that directly threaten crop productivity and food security (Allakhverdiev *et al*. 2000). According to a recent evaluation by the United Nations Food and Agriculture Organization (FAO),almost1.4 billion hectares of agricultural land,or 10.7% of the global land area, is affected by salinity (Harsha,2025). The agricultural land of the Sundarbans (West Bengal, India) was obstructed after the strike of cyclonic storm 'Aila' in 2009, due to the high salinity and alkalinity of the soil (Mandal *et al*. 2023).

In the Goshaba block of Sundarban, the average monsoon yield of rice was 4,219 kg/ha in 2008-2009. After 'Aila', this was decreased to 2,185 kg/ha in 2010-11, indicating an almost 50% reduction (Kar and Bandyopadhyay, 2015). Another super cyclone, Amphan, hit on the 16th of May, 2020, at 105 miles per hour and damaged agricultural land (Samanta *et al.* 2021).

Nearly 17,800 hectares of agricultural land are affected due to the entry of saline water fromthesea intothecultivated land, losing their fertility and making them uncultivable for the coming few years (Mandal *et al*. 2023). The annual cost of salt-related agriculture land degradation was estimated atUS$ 441 per hectare, yielding an estimate of global economic losses of US$ 27.3 billion per year (Qadir *et al*. 2014). It has been predicted that more than 50% of the arable land of the world will be salinized by the year 2050 (Qadir *et al*. 2014).

Rice is the primary food for over half the world's population, whereas China and India alone

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account for ~50% of the rice produced and consumed and provide up to 50% of the nutritional calories necessary for millions of Asians living in poverty.The countries that export rice include Thailand, Vietnam, Pakistan, the United States, India, Italy, Uruguay, China, the United Arab Emirates, Benin, Argentina, and Brazil, which trade for more than 90% of the global rice traded (Muthayya*et al*. 2014). Asia's production of rice is crucial for the world's food security and provides a major source of supply for the rise in global demand. Asian nations like Thailand, Vietnam, and India are expected to continue to dominate the worldwide rice trade (Bandumula, 2018). MTU1010 is an elite, high-yielding, short-duration rice variety that can be cultivated in different environmental conditions. Salinity has already damaged 16-20 million hectares of coastal land in South and Southeast Asia. In India, about three million hectares of agriculturallandare situated in West Bengal and Odisha, and the production of rice is decreasing by 12% for every unit of salt increase above a specific threshold (Chandrasekhar, 2018).Salinity stress harms plant growth and leads to an increase in the level of ROS, which results in oxidative stress, affecting the plant at the cellular and molecular levels (Ahmed *et al*. 2020). Bacteria followseveralmechanisms to mitigate salinity stress in plants.

The remediation of salinized agricultural land using low-cost and adaptable methods is a challenging goal for scientists. However, using plant growth-promoting microorganisms has great potential in eco-friendly and sustainable agriculture (Nadeem *et al*. 2014). The application of salt-tolerant plant growth-promoting bacteria (ST-PGPB) greatly enhances the growth of different crops in salinized agricultural land and potentially alleviates salinity's deleterious effects (Bakhshandeh *et al*. 2020). This research aimed to determine the influence of a nST-PGPB isolate inoculation on the growth of the MTU 1010 rice variety (Pratiksha W.B.) under salinity stress to assess its effect on the physiological responses of the rice variety.This was the first report on the growth promotion of the MTU1010 rice variety under salinity stress by application of any ST-PGPB isolate.

MATERIALS AND METHODS

Isolation of rhizospheric bacteria

For the isolation of bacteria, soil samples from the rhizosphere of vegetable crops were randomly collected. In this process, a pea (*Pisum sativum* L.) rhizosphere soil sample was collected from an agricultural field at Barajiakur (23.2886p N, 88.4719p E), Shantipur, Nadia, West Bengal, India. The electrical conductivity and pH of the sample were 0.82 dS/m and 6.8, respectively.The rhizospheric soil sample was serially dilutedand spread on Nutrient agar plates and incubated at 28±2 °C for 1-2 days. Bacterial isolates with different morphotypes were screened for NaCl tolerance and expression of in vitro plant growth promotion traits under the salinity stress. The mostpromising rhizobacterial isolate was selected and preserved in 50% glycerol at –80°C until further use.

Salinity tolerance study

Growth ofthe isolate on the Nutrient agar plate and in the Nutrient broth medium was tested with increasing NaCl concentration to identify the maximum tolerance level. For this, 1% bacterial inoculum was inoculated in the NB medium containing NaCl ranging from 100-1000 mM at pH 7.0 and incubated at 28±2 °C Growth was estimated by measuring OD at 600 nm at time intervals until the stationary phase. Salinity tolerance also studied by growing the isolate in the Complete medium [composition (g/l) K, HPO„ 0.5, MgSO„ 7H, O 0.4, NaCl 0.1, glutamine 1, mannitol 10, NH„ NO*ƒ* 1, pH 6.8].

Scanning electron microscopic of bacterial cells

Bacterial cells from both the control and salt-treated (at the highest tolerance level) sets were taken from the stationary phase, washed in phosphate buffer, fixed in glutaraldehyde, and observed under a scanning electron microscope, ZEISS at S.N. Bose Innovative Centre, University of Kalyani.

Antibiotic sensitivity assay

The sensitivity to antibiotics: ampicillin, chloramphenicol, neomycin, and streptomycin at

100 ppm concentration, was determined by agar cup assay on the bacterial lawn growing on NA. After incubation at 28±2 °C for a day, the antibiotic sensitivity and resistance were determined based on the presence and absence of a clear zone around each cup, respectively.

Morphological and biochemical characterization

The selected isolate was studied based on Gram staining, colony morphology on the NA plate, motility, optimal growth temperature and pH, casein hydrolysis, gelatin hydrolysis, urea production, catalase test, oxidase test, H_2S, acid and gas production in the presence of carbon sources such as glucose, maltose, lactose, sucrose, and mannitol. Biochemical characterization of the bacterial isolate was done according to Bergey's Manual of Determinative Bacteriology (Murray and Holt, 2005).

Estimation of exopolysaccharides (EPS) production and their functional group determination

The bacterial isolate was inoculated in the EPS production medium[composition (g/l): glucose 20, peptone 10, $MgSO_4$ 4, $CaCl_2$ 2, K_2HPO_4 1, pH 8] with low salt (0.5% NaCl, as control) and with high salt (at highest tolerance level, as salt stress) and incubated at 28±2 ^{0}C for three days(Arun *et al*. 2017). EPS was extracted from the cell-free supernatant by mixing two volumes of chilled ethanol. After centrifugation, the precipitate containing crude EPS was freeze-dried and quantified usingtheAnthrone method with glucose as a standard.The change of functional groups of the extracted EPS was interpreted through a Fourier Transform Infrared (FTIR) spectrometer (Hitachi F-7100) in the range of 4000-400 cm^{-1}.

Molecular identification and phylogenetic tree preparation

Bacterial genomic DNA was extracted from an overnight-grown bacterial culture using a QIAGEN genomic DNA isolation kit following the manufacturer's instructions. The *16S rRNA* gene was amplified using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The amplification reaction was performed in a thermocycler (Prime) according to the following program: one cycle of 4 min at 95 ^{0}C; 40 cycles of 1 min at 95 ^{0}C, 1 min at 55 ^{0}C, 1.5 min at 72^{0}C, and one final cycle of 7 min at 72 ^{0}C. The amplified product was visualized through 1% agarose gel electrophoresis, purified, and sequenced by the Applied Biosystem ABI Prism automated DNA sequencer at Barcode Biosciences, Bangalore-560077, India.

A homology search of the *16S rRNA* gene sequence was carried out against the GenBank database usingthe BLASTn tool, available on the NCBI platform. A phylogenetic tree was prepared through multiple sequence alignments using MEGA software version 11and the Neighbor-joining method.

Detection of in vitro plant growth-promoting activity

Production of indole acetic acid (IAA), siderophore, and amino cyclopropane decarboxylase (ACC) deaminase was tested under control and NaCl-supplemented NB medium.

IAA production test

The production of IAA by the bacterial isolate was tested using the Salkowski reagent. The bacterial isolate was grown in Nutrient broth supplemented with L-Tryptophan (500 ppm) up to the stationary phase. Then, the Salkowski reagent (2% 0.5 FeCl*ƒ* in 35% $HClO_„$ solution) was added to the cell-free supernatant ina 2:1 ratio and incubated for 30 min in the dark. The development of the pink color confirmed the production of IAA. The optical density of the color developed was measured at 540nm and compared with the standard curve of IAA.

Assay of siderophore production

Siderophore production was determined using the chrome azurol S (CAS) agar plate method (Datta and Chakrabarty, 2014). CAS agar medium was prepared in two steps: (i) to prepare blue dye solution, aqueous CAS solution (0.06 g in 50

ml of double distilled H, O) was mixed with iron solution (0.0027 g of FeCl*ƒ* 6 H, O in 10 ml of 10 mM HCl) in 5:1 ratio; this 60 ml solution was gently mixed with HDTMA solution (0.073 g in 40 ml of double distilled H, O).(ii) Separately, 900 ml of Complete medium was prepared and to this, PIPES buffer (30.24 g/l) was added and pH was adjusted 6.8 with 10N NaOH, then 20 g agar was added. After autoclaving and cooling to 45 °C, the blue dye solution was mixed into the medium and poured into plates. The bacterial isolate was inoculated at the center of the plate and incubated at 28 ÚCfor 3-5 days. Formation of an orange halo around the colony indicated the production of siderophore by the isolate.

Quantitative estimation of the siderophore was carried out using CAS reagent. The isolate was grown in the Complete medium for 3 days. The cell-free supernatant was mixed with CAS reagent in a 1:1 ratio, incubated for 30 min in the dark,and absorbance was read at 650 nm and compared with the standard curve of desferal.The catecholate (phenolate) type of siderophore was determined using an Arnow assay and thehydroxamate type using Atkin's method.

Assay for 1-aminocylopropane-1-carboxylate (ACC) deaminase activity

The bacterial isolate was at first grown in nitrogen-free Dworkin and Foster (DF) medium [composition (g/l): KH_2PO_4 4, Na_2HPO_4 6, $MgSO_4.7H_2O$ 0.2, glucose 2, gluconic acid 2, citric acid 2, $(NH_4)_2SO_4$ 2, $FeSO_4.7H_2O$ .001, trace elements 1ml; composition of trace elements (mg/l): H_3BO_3 10, $MnSO_4.H_2O$ 11.19, $ZnSO_4.7H_2O$ 124.6, $CuSO_4.5H_2O$ 78.22, MoO_3 10; pH 7.2±0.2 (at 25p C)] supplemented with ACC (3 mM) as a sole nitrogen source(Penrose and Glick, 2003). The bacterial cells were harvested by centrifugation, followed by washing in 0.1 M Tris-HCl, pH 7.6, to confirm that cells were free of the growth medium. The bacterial cells were suspended in 600 µl of 0.1 M Tris-HCl, pH 8.5, disrupted by adding toluene (30 µl) and vigorous vortexing. After mixing 200 µl of toluenized cell suspension with 20 µl of 0.5 MACC and incubating for 30 minat 30 °C, 1 ml of 0.56 N HCl was added and centrifuged for 5 min at 10,000 rpm. A reaction mixture devoid of ACC or cell suspension was employed as a control. After mixing 800 µl of 0.56 N HCl with 1 ml of supernatant, 300 µl of 0.2% 2,4-dinitrophenyl hydrazine in 2N HCl was added to the mixture. After adding 2 ml of 2 N NaOH and incubating the reaction mixture for 30 min at 30 °C, the optical density at 540 nm was measured to check for the production of á-ketobutyrate and compared with the standard curve of á-ketobutyrate.

Application of bacterial inoculum for the growth improvement of rice seedlings Inoculum preparation and seed treatment

The bacterial isolate was grown overnight in Nutrient broth, and the cell pellet was harvested and made into a cell suspension(4.8×10^8 CFU/ml) in sterile distilled water for further use in theexperiment.

Rice (*Oryza sativa* L.) MTU1010 seeds were disinfected by soaking in a 1% sodium hypochlorite solution for 2 min, rinsed 2-4 times with sterile distilled water, and 100 seeds were incubated overnight in the bacterial suspension (4.8×10^8 CFU/ml), and another 100 seeds were incubated in sterile distilled water. After incubation, seeds were transferred separately to Petri dishes (as controls and inoculated) containing two layers of moistened filter paper (100 seeds per Petri dish). The Petri dishes were kept at room temperature for four days, and the number of germinated seeds was recorded to calculate the germination rate.

Soil bed preparation for rice seedling trials

Four different sets of soil beds were prepared and designated as set-A control (only soil), set-B (soil inoculated with bacterial suspension), set-C (soil with 100 mM NaCl salt), and set-D (soil treated with 100 mM NaCl along with bacterial suspension). Each set of beds contained 300g of alluvial garden soil that was sterilized through an autoclave for 30 min, and after cooling, they were moistened with 30 ml of sterile distilled water or bacterial suspension 4.8×10^8 CFU/ml.

Greenhouse trials of the germinated seeds

After four days of incubation, germinated seeds of the control and inoculated sets were separately

transferred to a sterilized soil bed designated as control, inoculated, and kept in a greenhouse. The greenhouse temperature was maintained at 32±2 ^{0}C (daytime) and 28±2 ^{0}C (nighttime), the humidity was around 70%, and the CO_2 concentration was 400 ppm.

Rice seedlings trials

After 20 days of planting, the ten healthy seedlings were transferred to the set-A and set-C from the control set, and to the set-B and set-D from the inoculated sets.

Determination of seedling growth index

After 30 days from each set of experiments, five rice seedlings were randomly selected for the analysis of shoot length, root length, shoot fresh weight, root fresh weight, and number of leaves per seedling.

Determination of leaf chlorophyll content

The chlorophyll content of the selected rice seedlings from each set was estimated usingthe30-day-old third leaf from the tip. Fresh leaf disks were ground in 10 ml of 80% acetone and centrifuged at 2500 rpm for 10 min at 4 ÚC. The absorbance of the supernatant was measured at 665nm and 649 nm, respectively, using a spectrophotometer. The chlorophyll contents per gram leaf fresh water were determined using the following formulae:

µg Chl a per ml solution = (13.70) (OD_{665}) – (5.76) (OD_{649}).

µg Chl b per ml solution = (25.80) (OD_{649}) – (7.60) (OD_{665}).

Assay of antioxidant enzymes

The activities of the antioxidant enzymes,such as ascorbate peroxidase (APX) and catalase (CAT), were determined from the 30-day-old third leaf. The fresh rice leaf (0.5 g) was crushed in 10 ml chilled phosphate buffer (50 mM, pH 7.8). The supernatant was separated by centrifugation for 10 min at 10,000 rpm at 4 °C. To test APX activity, the reaction (2.85 ml of 100 mM phosphate buffer + 0.05 ml of L-ascorbate + 0.05 ml of H_2O_2) was initiated by adding 0.05 ml of fresh enzyme extract and followed by the immediate recording of a decrease in absorbance at 290 nm for 2 min. The APX activity per gram of leaf fresh weightwas measuredusing the molar extinction coefficient of 2.8 mM^{-1} cm^{-1} for the ascorbate;oneunitof enzyme equated to the amount of enzyme required to oxidize 1 nmol of ascorbic acid per min(Ashraf, 1989). To test CAT activity, 0.1 ml crude enzyme was mixed with 1 ml of 5.9 mM H_2O_2 in 1.9 ml of 50 mM phosphate buffer (pH 7.0). The optical density at 240 nm was read for a total of 2 min at a 20-second gap. A change of absorbance of 0.01 per min was equated to one unit of catalase activity (Nakano and Asada, 1987). Catalase was expressed as unit per mg protein.

Proline content assay

The proline content was determined from 30-day-old third leaves. Thefresh rice leaf (0.25 g) was crushed in 10 ml of 3% aqueous sulfosalicylic acid and incubated for 3 hr. After centrifugation at 2,000 rpm for 10 min, 2 ml of supernatant was added to 2 ml of glacial acetic acid and 2 ml acidic ninhydrin reagent (1.25 g ninhydrin in 30 ml of glacial acetic acid + 20 ml 6M H_3PO_4), boiled at 100 p C in a water bath for 1 hr. The reaction was stopped by placing it in an ice bath and adding 4 ml of toluene. This was kept at room temperature, and the absorbancewasread at 520 nm, using toluene as a blank. The proline concentration per gram of leaf fresh weight was determined based on the standard curve of L-proline(George*et al.* 2022).

Statistical analyses

The acquired data were presented in figures and tables as mean ± Standard Error (SE) from at least three replicates. Duncan's test determined those values with different letters significantly differed at p d" 0.05 (IBM-SPSS software).

RESULTS AND DISCUSSION

Isolation of halo-tolerant rhizobacteria

From the rhizosphere of pea plant, a number of rhizobacterial isolates were isolated and among

them, a isolate, designated as KUSP06 was selected since it showed maximum tolerance to NaCl (900 mM). On Nutrient agar medium, KUSP04 showed circular, smooth, convex, yellowish-white colonies with entire margins; but, the colonies were mixed with each other due to profuse production of exopolysaccharides. When the isolates were grown on NA plate with increasing NaClconcentration and it showed growth in the presence of 900 mM NaCl, but no growth was observed in 1000mM NaCl. However, in presence of the salt, the colony size was slightly reduced**(**Fig.1 a). The isolate showed almost similar growth pattern in control and 100 mM NaCl-supplemented NB medium and attained a stationary phase at 24 hr (Fig. 1b). However, in the presence of 900 mM NaCl, it showed slower growth, started the log phase at 24 hr, and attained maximum growth at 36 hr which was almost one-third of the growth found in the control set. However, the generation time of 8.46 hr and 6.85 hrin control and 900 mM NaCl-supplemented NB medium, respectively. The isolate also showed growth in the Complete medium upto 900 mM NaCl.

Change of cell morphology as a salt tolerance mechanism

Scanning electron microscopic image revealed that the cell morphology of the isolate KUSP04 altered in the presence of 900 mM NaCl. In the control set with 0.5% NaCl, all the cells were regular in shape with smooth cell surfaces (Fig. 1c). However, in the presence of 900 mM NaCl, the length of the cell increased by 23.28 %, but the cell's width decreased by 13.41% compared to that found in the control set (Fig. 1d). Under the salinity stress, the cell surface became rough, and the cell became filamentous with a scattered distribution. The results suggested that the isolate KUSP04 could resist a high salt environment by altering its cell morphology, and the rough cell surface might be due to the secretion of exopolysaccharides or capsules outside the cell.Similar results were found in the scanning electron microscopic view of the isolates *Bacillus megaterium,* where the cells started to form aggregation through EPS production (Chowdhury*et al*. 2011).

Characterization of the bacterial isolate

The bacterial cells were Gram-negative, rod-shaped. The isolate could tolerate temperatures of 25-40 ^{0}C and pH 5-9. It showed resistance against ampicillin and chloramphenicol at 100 ppm concentration but was sensitive to neomycin and streptomycin. The characteristic features summarized in (Table1) strongly recommend that isolate KUSP04 could be a member of the Enterobacteriaceae family (Murray and Holt, 2005).

Change of functional groups of exopolysaccharides under salt stress

Exopolysaccharide (EPS) production is one of the mechanisms for salt resistance where EPS can bind with sodium ions in the soil making them unavailable to the organism, EPS contributes to the formation of biofilm and these biofilms form a protection barrier around the root and maintain Na^{+}/K^{+} ratio in plant tissue (Liu *et al*. 2023). The EPS was extracted from the KUSP04 cells grown with or without salt stress. EPS showed increased sugar content upon exposure to salinity (205.72 mg/g from the 900mM NaCl-treated set in comparison to 105.58 mg/g from the control set). The FTIR analysis of extracted EPS showed the change of functional groups under salinity stress (Fig. 2). The broad stretching peak at 3451.73 cm^{-1} in the EPS of the control set and 3441.14 cm^{-1} in the EPS of salt treated set corresponded to the hydroxyl group (-OH), characteristics of carbohydrate ring (Lim *et al*. 2005). The small peaks at 2926.65 cm^{-1} and 2867.07 cm^{-1} in EPS from the control set and a single peak at 2928.75 cm^{-1} in EPS from salt stress are denoted as CH stretching mainly of methyl or methylene groups (Kalimuthu *et al*. 2023). The peak at 1625.38 cm^{-1} in the EPS of the control and 1640.07 cm^{-1} in the EPS of the salt-treated set was the stretching vibration of the acetyl group (Kalimuthu *et al*. 2023). The small peak at 1321.63 cm^{-1} was found only in the EPS of the salt-treated set and was absent in the control, corresponding to amino and peptide content (Gutierrez *et al*. 2008). The peak at 1150.90 cm^{-1} in the EPS of the control and 1090.58 cm^{-1} in the EPS of the salt-treated set corresponded to carbonyl groups stretching in the

Table 1: Morphological, physiological, and biochemical characteristics of the rhizobacterial isolate KUSP04

Characteristics	*Enterobacter* sp. KUSP04
Gram staining	Gram negative
Colony color on nutrient agar	Yellowish-white
Cell shape	Rod
Length	1.805-2.015µm
Width	0.461-0.781µm
Motility	+
Endospore	-
Temperature tolerance (optimum temperature)	25-40 °C (30±2 °C)
pH tolerance (optimum pH)	5-9 (7)
NaCl tolerance (optimum NaCl concentration)	Up to 900 mM (100 mM)
Indole production	+
Methyl red test	+
VP test	-
Nitrate reduction	+
Citrate utilization	+
Catalase	+
Oxidase	+
Urease	-
Amylase	+
Cellulase	-
Protease	+
Lipase	-
Gelatine hydrolysis	+
H_2S production	+
Acid and gas Production	+
Carbohydrate utilization	
Glucose	+
Maltose	+
Lactose	+
Sucrose	+
Mannitol	+

Note '+' indicates presence of the feature, '-'indicates absence of the feature

Table 2: Effect of inoculation of *Enterobacter* sp. KUSP04 on shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), number of leaves per seedling, chlorophyll and stress-related metabolites of the rice plant after 30 days

Treatments	Set-A (Control)	Set-B (KUSP04 inoculated)	Set-C (100mM NaCl treated)	Set-D (treated with 100mM NaCl + KUSP04)
SL (cm)	14.20 ± 0.52^{c}	19.56 ± 0.53^{d}	8.30 ± 0.43^{a}	11.30 ± 0.43^{b}
RL (cm)	4.80 ± 0.20^{c}	6.50 ± 0.28^{d}	2.03 ± 0.26^{a}	3.00 ± 0.28^{b}
SFW (mg)	40.71 ± 0.86^{b}	79.90 ± 0.80^{d}	20.54 ± 0.40^{a}	48.77 ± 0.65^{c}
RFW (mg)	10.66 ± 0.92^{b}	13.36 ± 0.71^{c}	5.66 ± 0.23^{a}	29.84 ± 0.84^{d}
No. of leaves/ seedling	3.33 ± 0.33^{ab}	4.00 ± 0.57^{d}	2.33 ± 0.33^{a}	3.00 ± 0.57^{ab}
Chlorophyll a (µg/g)	49.34 ± 0.33^{c}	55.85 ± 2.3^{d}	22.47 ± 1.8^{a}	40.52 ± 1.65^{b}
Chlorophyll b (µg/g)	29.89 ± 1.71^{b}	39.67 ± 3.83^{c}	15.89 ± 1.70^{a}	28.34 ± 1.39^{b}
Ascorbate peroxidase (U/g)	134.66 ± 2.40^{c}	154.66 ± 2.40^{d}	45 ± 2.51^{a}	78.00 ± 1.15^{b}
Catalase (U/mg)	14.70 ± 0.20^{ab}	21.00 ± 2.05^{b}	7.03 ± 0.26^{a}	11.23 ± 4.48^{a}
Proline (µg/g)	114.7 ± 0.60^{b}	72.26 ± 0.63^{a}	182.6 ± 1.13^{d}	157.3 ± 0.78^{c}

Mean ± standard error from three separate replicates. Duncan's test determined those values with different letters significantly differed at p d" 0.05.

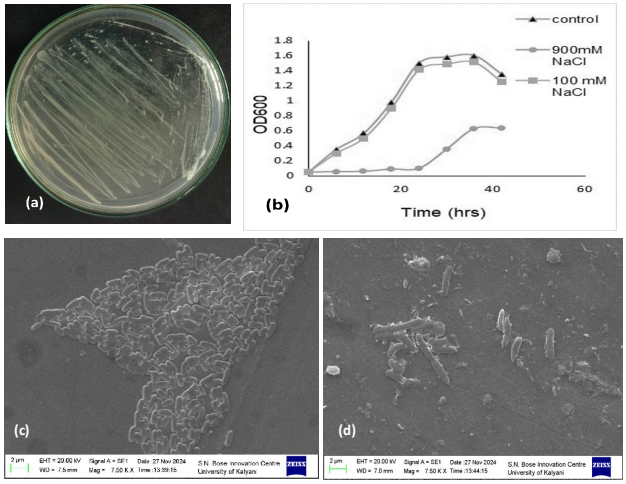

Fig. 1: (a) Growth of *Enterobacter* sp. KUSP04 on Nutrient agar plate supplemented with 900mM NaCl; (b) Growth kinetics of the isolate in Nutrient broth supplemented with two different concentrations of NaCl and the control set; (c) Scanning electron microscopic view showing cell morphology of the isolategrown in mediumwithout NaCl supplementationand (d) the presence of 900 mM NaCl (d)

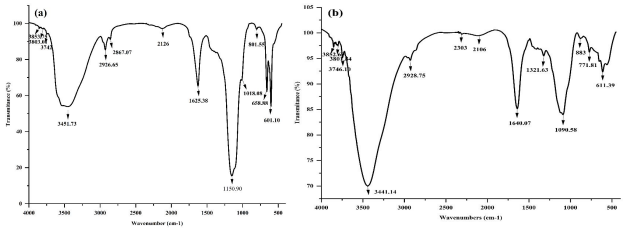

Fig. 2: Effect of salinity on variation of the functional groups of exopolysaccharides produced by *Enterobacter* sp. KUSP04, detected through FTIR analysis;EPS data were obtained fromtheculture grown in control set (a), andin900 mM NaCl-treated set (b).

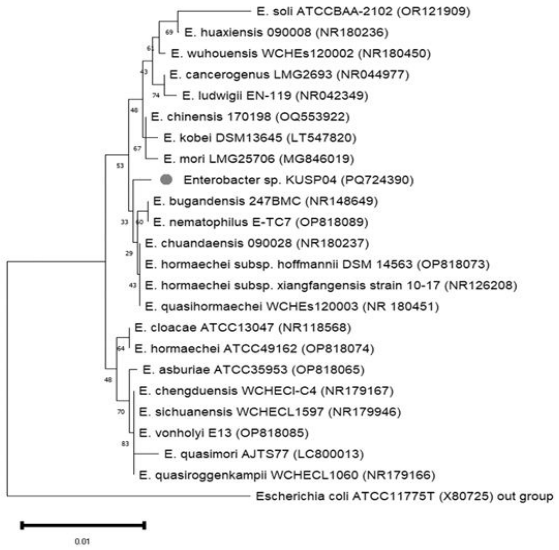

Fig. 3 : Phylogenetic tree based on *16S rRNA* gene sequence showing relatedness of the bacterial isolate KUSP04 and other *Enterobacter* species; the Neighbor-joining method generated the branching pattern using MEGA 11.

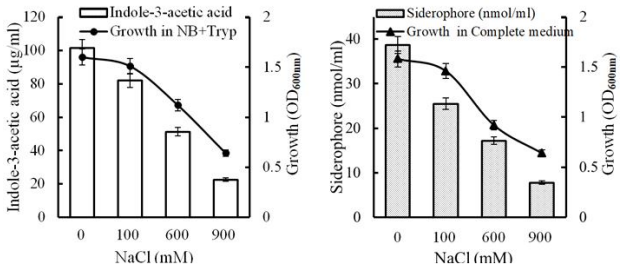

Fig. 4: Effect of sodium chloride on production of indole-3-acetic acid and siderophore by *Enterobacter* KUSP04;the bacterial culture was harvested at the stationary phase of growth

Fig. 5: Growth of 30 days rice MTU 1010 seedlings in the planting soil showing effect of bacterial inoculation to mitigate NaCl induced growth retardation

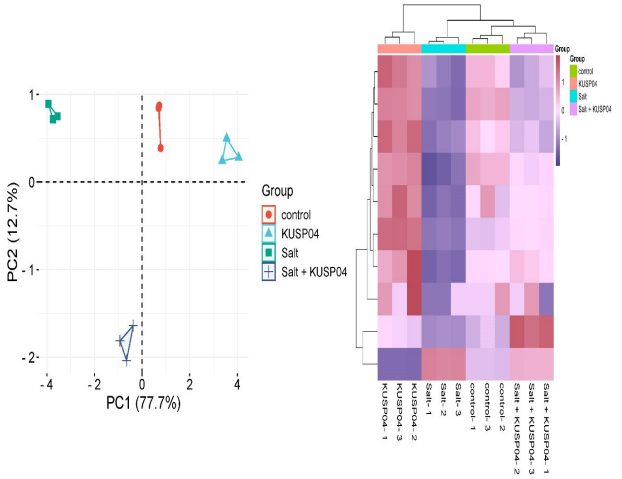

Fig 6 : Principal Component Analysis (a) and cluster heat-map analysis (b) of all variable data sets of rice in different treatment groups showing relatedness among the groups.

sugar backbone of polysaccharides (Cao *et al*. 2020). The peak at 1018.08 cm^{-1} only in the EPS of the control set indicated the presence of glycosidic linkage (C-OC-) (Ye *et al*. 2018). The peak in the region 600 cm^{-1} to 900 cm^{-1} (in EPS of both sets) denoted polysaccharides with aromatic modification (Wang *et al*. 2015).

Molecular identification

A ~1.5 kb PCR product was obtained using the primer pair 27F and 1492R. The PCR product was sequenced, and the BLASTn search of the *16S rRNA* gene sequence results showed that the isolate KUSP04 had more than 99.0% similarity with different *Enterobacter* species. However, it showed maximum similarity (99.49%) with *E. quasihormaechei*WCHEQ120003, *E. chuandaensis*090028, and *E. hormaechei*subsp. *xiangangensis*10-17. Based on the results, the isolate was named *Enterobacter* sp. KUSP04. Using the Neighbor-joining method, the phylogenetic position of the isolate KUSP04 was analysed, and it was placed in the same clade as the three *Enterobacter* species (Fig.3). The *16S rRNA* gene sequence was deposited in GenBank under the accession number PQ724390.

In-vitro plant growth promoting attributes

Enterobacter species isolated from rhizosphere soil can influence plant growth in several ways: nitrogen fixation, phosphate solubilization, production of IAA, ACC deaminase, siderophore, ammonia, and HCN, and tolerance to biotic and abiotic stress (Sarkar et al., 2018).The isolate KUSP04 was also able to produce indole-3-acetic acid andsiderophore,both in the presence and absence of NaCl. However, with the increase of NaCl concentration from 100 to 900 mM, their production decreased (Fig.4). IAA is a crucial signaling molecule in plant-microbe interactions. It regulates cell division and elongation of lateral roots and plays a major role in stress tolerance. Under the salinity stress of soil, plants produce lower levels of IAA, and as a result, the overall growth and development of plants are affected. Production of IAA by rhizospheric bacteria, even under salinity stress, can improve the root architecture and plant biomass. Due to the increase in surface area, plants will be able to extract more nutrients from the soil. It was reported that the IAA-producing *Enterobacter* sp. CM94 significantly promoted chickpea growth under salt-stress conditions (Sharma *et al*. 2023). Iron is an essential element for all living organisms, but under neutral pH in the presence of oxygen, it precipitates in an insoluble form. To face the challenge, microorganisms secrete siderophores to chelate ferric iron and take up iron into the cell. Some bacterial siderophores can be utilized by the plant, and siderophores act as biocontrol agents, depriving the pathogen of iron. *Enterobacter* species produce two types of siderophore: enterobactin (a phenolate type) and aerobactin (a hydroxamate type). The isolate KUSP04 produced fluorescent green pigment in the complete medium after three days of growth, and both catecholate and hydroxamate types of siderophore were detected in the broth culture.Siderophore produced by endophytic *Enterobacter* sp. YG-14 mediated arsenic mobilization and helped in phytostabilization (Chen *et al*. 2024). The bacterial isolate KUSP04 produced both phenolate and hydroxamate siderophores, which made the isolate an efficient bio-inoculant agent for plant growth promotion.
Aminocyclopropane carboxylate is the precursor of ethylene, and microorganisms produce ACC deaminase reduces the toxic effect of ethylene through the degradation of ACC. ACC deaminase activity has been reported from several beneficial rhizospheric bacteria, including *Enterobacter* sp. Enhanced ACC deaminase activity has been reported from endophytic *E. ludwigii* under salt stress (Dolkar *et al*. 2018).The isolate KUSP04 produced a sufficient amount of ACC deaminase (77.25±2.15nmol á-KB /mg/h).

Effect of Enterobacter KUSP06 on rice seed germination and growth promotion

NaCl (100mM) had a negative effect on seed germination of the rice MTU1010 variety showing only 15.66 ± 0.88% of seed germination. Inoculation of KUSP04 improved seed germination showing19.65% germination under the NaCl stress and 68.14% germination in absence of NaCl stress. Under normal condition, KUSP04 treatment improved length of plumule and radical up tothreefold and 3.32-fold, respectively.

Treatment of the isolate KUSP04 in the planting soil could mitigate toxic effects of salinity stress on rice MTU1010 growth (Fig.5). Results depicted that the rice MTU1010 variety could survive in the presence of 100mM NaCl but shoot and root length, shoot and root fresh weight, and number

of leaves per seedling reduced 41.5%, 57.7%, 49.5%, 46.9% and 0.3%, respectively under the salinity stress as compared to control (Table2). This growth retardation effect of NaCl was recovered up to some extent by the application of KUSP06 (36.1%, 47.8%, 137.4%, 427.2% and 28.8% increase in shoot and root length, shoot and root fresh weight, and number of leaves per seedling, respectively). However, in absence of NaCl stress, the isolate KUSP04 inoculation could significantly ($p < 0.05$) improve rice growth (37.7%, 35.4%, 96.3%, 25.3% and 20.1% increase in shoot and root length, shoot and root fresh weight, and number of leaves per seedling, respectively).The similar growth improvement of rice plants was observed when inoculated with *Enterobacter asburiae* D2 resulting34.7% increase in shoot length and 57.1% increase in root length in absence of NaCl supplementation, and 18.1% increase in shoot length and 25.9% increase in root length in presence of 150mM NaCl supplementation (Ning *et al*. 2024).

As compared to the uninoculated control, the content of chlorophyll a and b decreased by 54.5% and 46.8% in plants under the 100 mM NaCl stress. However,KUSP04 inoculation retrieved chlorophyll a and b content of the NaCl stressed plants with increase of 80.3% and 78.4%, respectively (Table2).The activity of antioxidant enzymes such as catalase and ascorbate peroxidase was observed as highest in plants inoculated with the isolate as compared to control. The protective antioxidant enzymes were decreased due to prolonged salt stress, and the rice variety almost died or senescence under salt stress compared to the control. It was observed that the rice leaf became brown or dry and wilted or curled under salt stress, as death progressed, necrotic patches developed. The roots became brown or necrotic, and their growth was reduced, affecting overall growth of the rice plants. Highest proline content was observed in the salt treated plants and lowest in KUSP04 treated plants indicating bacteria inoculation did not hamper normal activity of the plant.

A principal component analysis was built along with the morpho-physiological, biochemical, and antioxidant traits of the rice plants under control, salinity stress, KUSP04 inoculated, and combined (KUSP04 + salinity) treatment groups, and showed that PC1 and PC2 were able to account for 77.7% and 12.7% of the variance in the data set, respectively. The combined treatment group showed a more significant difference with the salinity treatment group and the absence of salinity, the isolate KUSP04 treated group also showed a significant difference, but less concerning the salinity treated group (Fig.6a). Moreover, the cluster analysis revealed that four treatment groups were grouped into three main clusters. Remarkably, the combined (KUSP04 + salinity) treatment group showed a close relationship with the control group (Fig. 6b). Therefore, it can be inferred that inoculation of the isolate KUSP04 might ameliorate salt tolerance status of rice variety MTU1010through production of PGP compounds as well as array of other stress enzymes such as lowering ethylene levels in plantsthrough the production of ACC deaminase.

Application of *Enterobacter hormaechei* MF 957335 increased fresh biomass, shoot and root length, and fruit size of tomato plants in the presence of 250 mM NaCl (Ranawat *et al*. 2021). *Enterobacter ludwigii* SRI-211 and *E. ludwigii* SRI-229 significantly enhanced the growth of rice in terms of the tiller numbers, stover and grain yields, total dry matter, root length, volume, and dry weight over the uninoculated control (Gopalakrishnan *et al*. 2012). Ion homeostasis and ROS scavenging are the possible mechanisms employed by bacteria to mitigate salinity stress in rice plants. Some salt-tolerant bacteria upregulate rice plant ion transporters such as Na^+/H^+ antiporters, which help the extrusion of excess Na^+ from the cytoplasm to vacuoles (Gupta *et al*. 2023). Beneficial bacteria upregulate high-affinity K^+ transporters, which help in the uptake of K^+ rather than Na^+, thereby maintaining a suitable K^+/Na^+ ratio. Some rhizobacteria produce EPS, which can bind to Na^+ ions, making them unavailable to plants (Bhagat *et al*. 2021). Bacterial inoculation increases antioxidant enzyme levels in rice plants such as catalase and superoxide dismutase, reducing oxidative damage (Gupta *et al*. 2023). *E.asburiae* D2 inoculation of rice plants increases ion intake during salt stress, reducing Na^+ accumulation and increasing K^+ uptake, hence sustaining the K^+/

Na^+ratio, which is essential for cellular function and increases the activities of antioxidant enzymes such as peroxidase and catalase in rice plant thereby enhancing the ROS scavenging with the reduction of oxidative stress (Ning *et al*. 2024). The survival ability of the isolate in the rice rhizosphere was tested by recovering the isolate from the bacteria treated roots and checked their morphological and biochemical characteristics.

CONCLUSION

From the present investigation, it was concluded that *Enterobacter* sp.KUSP04 showed moderate resistance to NaCl and secured the rice plants as opposed to the inhibitory outcome of NaCl with the help of its plant growth-promoting activity (IAA production, siderophore production, ACC deaminase activity, exopolysaccharide production). Inoculation of this bacterial isolate successfully increased the growth of rice plants in terms of biomass fresh weight, length, chlorophyll content, and level of antioxidant enzymes,and decreased the oxidative stress undersalt stress. So, the bacterial isolate KUSP04 might be used as a potential bio-inoculant for rice plants to reduce the effects of saline soil.

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DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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