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Diazotrophic Endophytic Bacterium *Alcaligenes* sp. KA31 and its Role in Promoting the Growth of Wheat (*Triticum aestivum* L.*)* Under Saline Conditions

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ABSTRACT

Intensification of food production with an increasing world population, to achieve food security has become one of the major agricultural challenges for a sustainable future. Salinity is the second most abundant threat after drought which causes disturbance in the symbiotic performance of plants and increases their susceptibility to detrimental soil-borne pathogens. In the present study, endophytic diazotroph KA31 isolated from roots of wheat plant was characterized and identified as *Alcaligenes faecalis*. The bacterial endophytic isolate produced indole acetic acid (IAA), and siderophore along with the potential to solubilize phosphorous (P). KA31 was also able to fix nitrogen (N) as evaluated through acetylene reduction assay and *nifH*-PCR assay. Recolonization ability of *A. faecalis* in the wheat seedlings was studied under laboratory conditions and it was demonstrated that KA31 was able to establish an endophytic association with the host plant. Furthermore, in the pot experimental study, *A. faecalis* KA31 significantly improved the growth parameters of wheat plants under saline conditions as compared to untreated control plants. Presumably this is the first study that reports *A. faecalis* as salt tolerant diazotrophic plant growth promoting endophyte (PGPE) and overall the work depicts that novel bioformulations developed using them can be effective for wheat crops grown in saline soils.

Key words: Salinity, Nitrogen fixation, Endophytes, Agriculture, Microorganisms, Sustainability

INTRODUCTION

Wheat is one of the most important staple crop in temperate countries and it is also the world's most consumed cereal grain (Shewry and Hey 2015). Among thousands of known varieties of wheat, only three are considered to be most significant i.e., common wheat (*Triticum aestivum*), durum wheat (*T. durum*) and club wheat (*T. compactum*). *T. aestivum* is primarily utilized as flour for the production of bread, and a variety of other baking products. The rest, mostly include durum wheat, which is milled to produce semolina (coarse flour), the main raw material of pasta (Igrejas and Branlard, 2020). Though amplification in global wheat production has been observed in the last few years, yet the latest forecast for world wheat production stands at 776.7 million tonnes as per the report of the Food and Agricultural Organization of United Nations (https://www.foodingredientsfirst.com/news/fao-food-price-index-continues-to-rise-in-september.html). Further, the increasing need for food, feed, and crop yields with high fiber content paralleled by injudicious use of chemicals, ill-agricultural practices, and climate change is leading to unusual soil erosion on arable lands (Frona et al. 2019). Among the multiple abiotic stresses faced by plants, salt stress is one of the major detrimental plant growth limiting factor generating marginal lands and reducing the yield and quality of crops (Bharti et al. 2016, Mittler 2006, Bokhari et al. 2019, Arora et al. 2020). Henceforth, it is prime time to develop sustainable agricultural approaches that would mitigate the negative impacts of agrochemicals and unhealthy agricultural practices leading to a better sustainable future (Elkoca et al. 2010). Plants and microorganisms are well known for their symbiotic alliance and this relationship efficiently ameliorates agricultural traits encompassing improved soil health as well as heightened plant growth and development (Bokhari et al. 2019, Tewari and Arora 2016, Hemida and Reyad 2018). Thereby, the helpful microbial symbionts of plants can be exploited as one of the remarkable practices with the intent of advancing sustainable agricultural productivity and lessening the impacts of climate change and abiotic stresses (Vandana et al. 2020).

Multifaceted population of microorganisms known as plant growth-promoting rhizobacteria (PGPR) live in close association with roots and are capable of affecting the health and resilience of plants under both hostile and unhostile conditions. These microbes can either establish themselves in the rhizoplane, rhizosphere, phyllosphere or can also reside inside the plant tissues in the form of endophytes. According to Petrini (1991), the term endophyte is signified as a “large and diverse group of microbes that inhabit plant organs at some time in their life and colonize internal tissues of plants without causing any harm to their hosts”. These beneficial bacteria can either house in roots, stems, or leaves and have been extensively studied in recent years for their ability to solubilize nutrients, synthesize plant hormones such as indole-3-acetic acid (IAA), secrete siderophores, and confer tolerance against biotic and abiotic stresses (Rosenblueth and Martínez-Romero 2006, Gaiero et al. 2013, Lebeis et al. 2015, Khare et al. 2018). Additionally, certain endophytic bacteria known as diazotrophs can provide nitrogen to the plants and contribute to biological nitrogen fixation (BNF) by converting dinitrogen gas (N_2) into usable forms such as nitrate and ammonium (Santi et al. 2013). Colonization of plants by endophytic genera such as *Gluconobacter, Azoarcus, Burkholderia, Klebsiella, Pantoea, Herbaspirillum, Rahnella,* and *Pseudomonas* have been already demonstrated in several reports (Elbeltagy et al. 2001, Iniguez et al.,2004, Momose et al. 2009, Knoth et al. 2012, Kandel et al. 2017). Although various studies emphasizing on bacterial endophytes of wheat were carried out in the past (Qiao et al. 2006, Herrera et al. 2016, Kiyani et al. 2019), but so far no reports on salt-tolerant diazotrophic bacterial endophytes are available which show synergistic impact on growth and stress resilience in plants. The present study highlights the screening of endophytic bacterial community in wheat along with assessment of plant growth promoting and salt stress resistance traits of selected isolates. Applying such potent diazotrophic endophytic isolates as bio-inoculants might improve the productivity of plants as well as remediate the salt-stressed marginal lands in a sustainable and cost-effective manner.

MATERIAL AND METHODS

Study site, collection of root samples, and isolation of endophytes

The present work focused on the semi-arid areas of central Uttar Pradesh, India and the districts chosen were Lucknow (26°43 N and 80°51 E) and Kanpur (26°11.26 N and 80°10.05 E). For the enumeration of bacterial root-endophytes, healthy wheat samples were randomly collected at flowering stage from salt stressed soil. Plants were carefully uprooted, packed in sterilized bags, immediately transported to the laboratory for isolation of endophytes within 24 h of collection.

To isolate the endophytes, firstly the roots were washed under running tap water to remove adherent soil, and then surface sterilization was done by immersing the roots in 70% ethanol for 1 min (Anjum and Chandra 2015). After washing with sterilized distilled water, roots were treated with 0.1 percent mercuric chloride for 10 min followed by six times washing with sterilized distilled water (Rashid et al. 2012, Hallmann et al. 2006). To standardize the efficiency of surface sterilization, some of the root tissue samples were placed on nutrient agar (NA) medium plates, and bacterial growth around the root segments was observed. The plant tissues were sectioned into small pieces and homogenized using a sterile mortar. Suspensions were serially diluted to 1×10^{-3} and aliquots (0.1 ml) of each dilution were inoculated in triplicate onto NA and yeast extract mannitol agar (YEMA). The plates were then placed for incubation at 28ºC for 24-48 hours. Morphologically different bacterial colonies were repeatedly purified on NA plates to obtain the monocultures of isolates. The cultures were further maintained in 25% glycerol culture stocks at -80ºC for further use. Isolates were characterized as per Bergey’s Manual of Systematic Bacteriology (Garrity 2005).

Plant growth promoting (PGP) traits

The characterization of the endophytic isolates for the exhibition of plant growth promoting traits was done by performing qualitative and quantitative analysis. Phosphate solubilization property was checked by inoculating the isolates on Pikovskaya agar and after incubation, the plates were observed for the appearance of transparent "halos" (Pikovskaya 1948). The solubilization of K by isolates was checked on modified Aleksandrov agar medium using mica (potassium aluminum silicate) as the source of insoluble K and phenol red as acid-base indicator dye. The spot inoculated plates were placed for incubation at 30ºC for 48–96 h and the clear zone along with color change was noted for K solubilization (Rajawat et al. 2016). Zinc solubilizing activity was determined on Tris-minimal medium supplemented with 1% of insoluble zinc compounds (ZnO, ZnS, $Zn_3(PO4)_2$) according to method described by Fasim et al. (2002). Bacterial cultures were spot inoculated on zinc medium plates, incubated for 48–96 h at 30ºC, and solubilization index was estimated by measuring the transparent zone around the colonies. Indole-3-acetic acid (IAA) synthesis was quantified by colorimetric assay, using Salkowski's reagent with a few drops of orthophosphoric acid and measuring the development of color at 530 nm (Ahmad et al. 2008). The concentrations of IAA were expressed as µg/mL and determined by using a standard IAA curve. Siderophore production was evaluated on both Chrome Azurol S (CAS) agar plates as well as in broth by using microtiter plates as per CAS-Shuttle assay and was expressed as percent siderophore unit (psu) (Schwyn and Neilands 1987, Arora and Verma 2017).

Screening of nitrogen fixers

Nitrogen fixation

The isolates were preliminary evaluated on the basis of their nitrogen-fixing ability by growing them on Nitrogen free Jensen's medium (20g sucrose, 1g K_2HPO_4, 0.500g $MgSO_4$, 0.500g NaCl, 0.100g Fe_2S0_4, 0.005g $Na_2MoO_4.2H_2O$, 2.000g $CaCO_3$, and 15.000g agar) containing bromothymol blue (BTB) as a color indicator (Okon et al. 1977). The plates were kept for incubation for 48-72 hours and the color alteration of media from greenish blue to dark blue indicated nitrogen-fixing activity.

Acetylene reduction assay

The assay was performed to assess the nitrogen fixation ability of bacterial isolates quantitatively. Freshly grown cultures of selected isolates were taken and inoculated into tubes containing 50 ml of N-free modified Burk's medium (Kifle and Laing 2016). The medium was incubated at 28^{o}C for 5 days to obtain the aliquots (0.1 OD at 610 nm) till the exponential phase. The cotton plugs of the vials were replaced with Suba-Seal; the air inside the headspace was replaced with acetylene (10% v/v) and incubated further for 6 h at 28^{o}C. Nitrogen fixation was determined by measuring the ethylene production and the analysis was accomplished by gas chromatography (Agilent 7890A GC System) using an HP-5 column equipped with a flame ionization detector (FID). All the experiments were conducted in triplicates and the concentration of ethylene was further calculated. In total ten isolates were screened for their potential to fix N and based on the same endophytic isolate KA31 was selected for further characterization and experimental work.

Genotypic characterization of selected isolate and phylogenetic analysis

Molecular identification of the screened isolate was done by 16S ribosomal RNA (rRNA) gene sequencing using the primers 243F (5' - GGATGAGCCCGCGGCCTA-3') and 1378R (5'-CGGTGTGTACAAGGCCCGG-3'). The reactions were performed at a final volume of 100 µL and the cycling conditions included the following steps: initial denaturation for 2 minutes at 95°C; 35 cycles with a denaturation step for 30 seconds at 95°C, 52°C for 30 seconds, 72°C for 2 minutes; and 72°C for 15 minutes as the final extension time. The amplification products were visualized by agarose gel electrophoresis (1.2%) on a gel documentation system and gel images were digitalized. Purification and sequencing of obtained PCR products were done and nucleotide sequences were analyzed with Basic Local Alignment Search Tool (BLAST) against the reference sequences of the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1997). Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) implemented in the program MEGA software, version 6 (Kumar et al. 2018).

PCR amplification of *nifH* gene

The selected isolate KA31 was also analyzed for the presence of *nifH* gene by polymerase chain reaction (PCR). Genomic DNA was extracted according to the chloroform/isopropyl alcohol method and primers used in the study were 19F (5'-GCIWTYTAYGGIAARGGIGG -3') and 407R (5'-AAICCRCCRCAIACIACRTC-3'). The PCR conditions maintained were as following: pre-run for 2 min at 95^{o}C, 40 cycles of initial denaturation for 30 sec at 94°C, annealing for 1 min at 50°C, extension for 0.5 min at 72°C, followed by a post-run for 5 min at 72°C (Ueda et al. 1995). Obtained PCR products were processed for agarose gel electrophoresis for the separation of DNA fragments and further analysis.

Halotolerance assay

Salt sensitivity test was done by inoculating log phase culture of the selected isolate to nutrient broth medium supplemented with various concentrations of NaCl (0-8%). Tubes were incubated at 28°C for 36h and the optical density (600 nm) was measured by using ThermoScientific™ Evolution 201 UV–Vis spectrophotometer at every 4 hr up to stationary phase (Khare et al. 2011).

Determining the colonization ability of selected isolate

The selected endophytic isolate was assessed for its ability to infect and colonize the plantlet tissue (Hernawati et al. 2011). Salt tolerance property of wheat variety (Annapurna PBW-343) was checked by water plate agar assay and the variety was selected because the local farmers commonly used it for the cultivation of wheat. The wheat seeds were surface sterilized by treating them with sodium hypochlorite (NaOCl) solution for 5 min. Afterwards the seeds were treated with the freshly grown culture of the selected isolate and were allowed to grow on petri dishes for two weeks under aseptic conditions. The experiment was done in triplicates and roots taken from the germinated plants were processed for the re-isolation of endophytes using the same protocol as described above. The obtained bacterial colonies were then identified by 16S rRNA sequencing and matched with the lab culture of the selected isolate (Fatima and Arora 2021).

The plant microbial interaction was also tested by means of scanning electron microscopy (SEM). Preparation of the specimens were carried out by fixing the root tissues with the help of 2·5% solution of glutaraldehyde in phosphate-buffered saline for 30 min. Samples were then processed for dehydration in ethanol solutions of increasing concentrations and after the final dehydration step in absolute ethanol, the roots were cut and dissected for cross-sections (Akhdiya et al. 2014). The sectioned samples were then attached to stub specimens with double cello-tape, coated, and were examined at magnification 750x and 1000x by using SEM (Model: JEOL, JSM 6490LV) at University Sophisticated Instrumentation Unit (USIC) BBA University, Lucknow.

Pot experiment

Pot trials were conducted for two consecutive years (2019 and 2020) during the rabi season i.e., November-February in earthen pots sized 15 × 11 × 11 cm. The pots were filled with sterilized and non-sterilized saline soil (EC 8.5 dS/m) that was collected from adjoining rural areas of Lucknow, India (26.72°N, 80.84°E). Chemical properties of the soil including pH, electrical conductivity (EC), organic carbon, available N, P, and K were checked as per the standard protocols (Jackson et al., 1973). Experiments were conducted in five replicates with following sets of treatments: (i) untreated control (ii) seeds + KA31. Wheat seeds of Annapurna-PBW 343 were selected for the study and checked for salt tolerance by water agar plate assay. Prior to sowing, the seeds were surface sterilized with NaClO (diluted 1:5 in sterile distilled water) for 5 min; washed with 1% Tween for 5 min followed by thorough rinsing with deionized water (Gamalero et al. 2004). Aqueous solution of 1% carboxy methyl cellulose (CMC) was autoclaved and was used as binding agent to effectively coat the sterilized seeds (4 ml CMC with 1 g seed) (Weller and Cook 1983). Surface sterilized seeds were immersed in bacterial suspension (1×10^8 CFU/mL) of the selected isolate and CMC solution was added. Dipped seeds were kept overnight (10-12 h), air-dried for 3-4 h, and were sown in pots (10 in each). Seeds without treatment were used as an experimental control. Plants were carefully uprooted 60 days after sowing (DAS) and plant growth parameters such as root length, shoot length, plant dry weight and fresh weight, spike length, and the tiller numbers were evaluated.

Statistical Analysis

All data were statistically analyzed using MS excel 2010 and statistical package software for social sciences (SPSS) 2016. The comparisons between growth parameters of the treatments were carried out using the Student's t-test at a probability of alpha = 0.05.

RESULTS

Isolation of endophytes and their plant growth promoting characterization

A total of ten morphologically distinct colonies of the isolates i.e., KA14, KA22, KA27, KA31, KA32, KA35, LK7, LK11, LK16, LK19 were obtained from the root tissues of wheat and were biochemically characterized. The results indicated that diverse endophytic bacterial populations inhabited the roots and most of them were found to be Gram negative, motile, rod-shaped, positive for, citrate, catalase, and negative for indole, urease, gelatinase, and methyl red (MR) test. Bacterial endophytic isolates were also screened for multiple plant growth promoting traits and were found positive for one or more activities (Table 1). The nutrient chelation properties of all the isolated endophytes were checked and the results showed that only five of them were able to solubilize phosphate. Solubilization of Zn and K were also assayed and four isolates (KA14, KA22, KA31, LK11) gave positive results for Zn whereas only one isolate (LK11) solubilized K. The results of the study indicated that L-tryptophan amended as a precursor in NB medium was converted to IAA. All the bacterial endophytic isolates except two (KA32 and LK11) were IAA producers and maximum production was by KA31 (27 μg ml^{-1}) and minimum by LK19 (11 μg ml^{-1}). In the study, siderophore production was observed only in two isolates (LK7 and KA31); however, comparatively isolate KA31 was found to be the most potent siderophore producer showing 24.15 psu through CAS shuttle assay.

Screening of diazotrophic endophytes

Nitrogen fixation was assayed qualitatively on Jensen's medium and isolates were observed for growth characteristics and color change. Only three isolates (KA14, KA31, LK16) were able to grow on the nitrogen-free Jensen's medium and the bacterial colonies appeared to be mucilaginous and translucent in nature. The color alteration due to the increase in pH of the medium due to ammonia production (Table 1) indicated that these endophytic bacteria were able to fix nitrogen. N fixation efficiency of these three

Table 1. Plant growth-promoting attributes of salt tolerant bacterial endophytes

Strains	**Solubilization**			**Production**		**Salinity (%NaCl)**				**N_2 fixation (on N free medium)**	**ARA (nmole C_2H_4 h^{-1} culture^{-1})**
	Zinc (zsi)	**Phosphate (psi)**	**Potassium**	**IAA (μg/ml)**	**Siderophore (psu)**	**2**	**4**	**6**	**8**		
KA14	+	+	-	+	-	+	+	-	-	+	27.43
KA22	+	-	-	+	-	+	+	+	-	-	-
KA27	-	+	-	+	-	+	-	-	-	-	-
KA31	**+** **(2.11 +0.2)**	**+** **(2.5+0.3)**	**-**	**++** **(27+ 0.51)**	**++** **(35.3+1.5)**	**+**	**+**	**+**	**+**	**+**	**29.806**
KA32	-	-	-	-	-	+	+	-	-	-	-
KA35	-	+	-	+	-	+	-	-	-	-	-
LK7	-	-	-	+	+	+	+	-	-	-	-
LK11	+	-	+	-	-	+	+	-	-	-	-
LK16	-	-	-	+	-	+	+	-	-	+	26.95
LK19	-	+	-	+	-	+	-	+	-	-	-

+ = positive, ++ = strongly positive, - = negative, psi= phosphate solubilisation index, zsi= zinc solubilisation index, psu= percent siderophore unit. Results expressed as mean ± S.D (n = 3) isolates was also determined in terms of nitrogenase enzyme activity and expressed as ARA activity. The maximum value of the ethylene production was obtained by isolate KA31 i.e., 29.806 ± 2.4 n moles C_2H_4 culture^{-1} h^{-1}. Isolate KA14 showed 27.43 ± 3.9 n moles C_2H_4 culture^{-1} h^{-1} and the lowest ARA activity was seen in the case of LK16 as depicted in Table 1.

Molecular identification of bacterial endophytic isolate and *nifH* gene amplification

The screened isolate KA31 was genotypically characterized by 16S rRNA sequence and BLAST analysis showed high sequence similarity (99.2%) with *Alcaligenes faecalis* strain NBRC 13111 (Accession No. NR_113606.1). The phylogenetic tree was constructed and the closest evolutionary distances were indicated as evident in Figure 1**.** Gene sequence data was submitted to the NCBI GenBank database with Accession No. OL423363**.** PCR amplification of template DNA using forward and reverse primers of *nifH* led to the generation of ~390 bp sized gene fragments and this confirms the presence of the respective gene in KA31 (Fig. 2). The results suggest that the bacterium possesses metabolic properties of fixing the N_2 and thus have the potential to promote the plant growth.

Salt tolerance properties of KA31

KA31 was checked for cell growth and survival at various salt concentrations (2%-8%) and results showed that the bacterium tolerated salinity stress up to 6% NaCl. No change in growth pattern of the isolate was observed up to 2% NaCl concentration, however salinity above 4% considerably reduced the growth rate (Fig. 3).

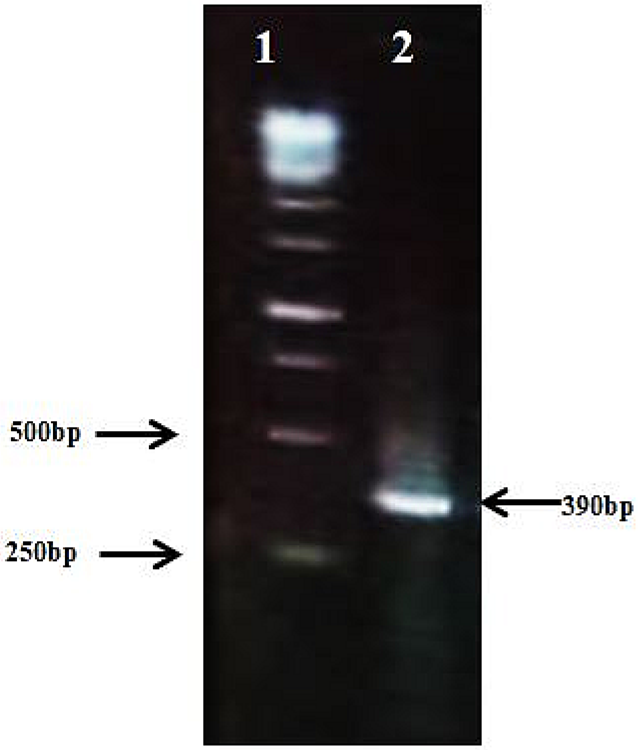

Figure 2. PCR amplification of an internal *nifH* fragment of ~390 bp (indicated by the arrow) 1-Ladder 2- Isolate KA31

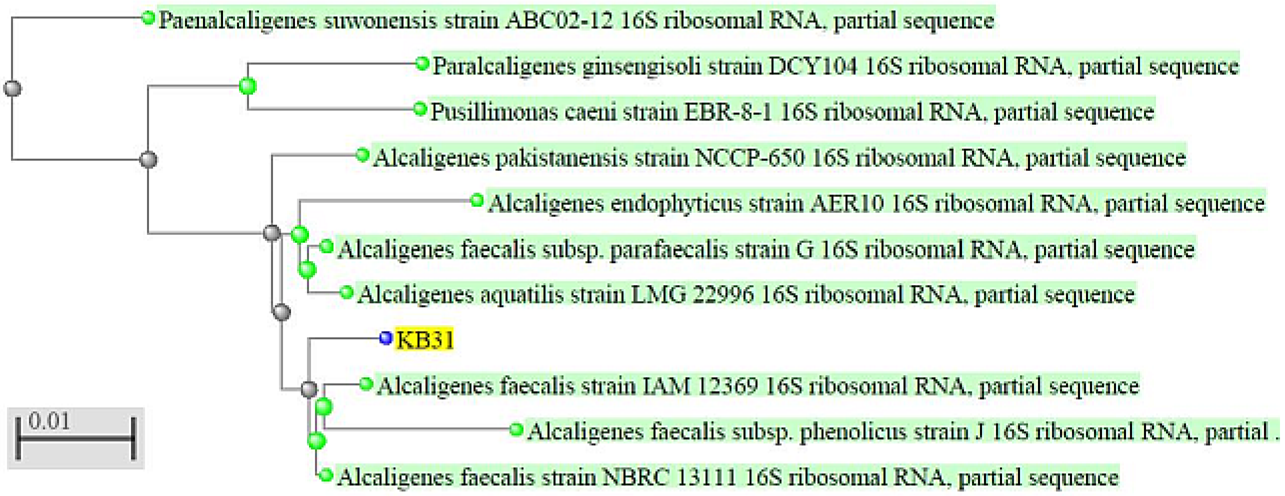

Figure 1. Phylogenetic tree of the isolate KA31

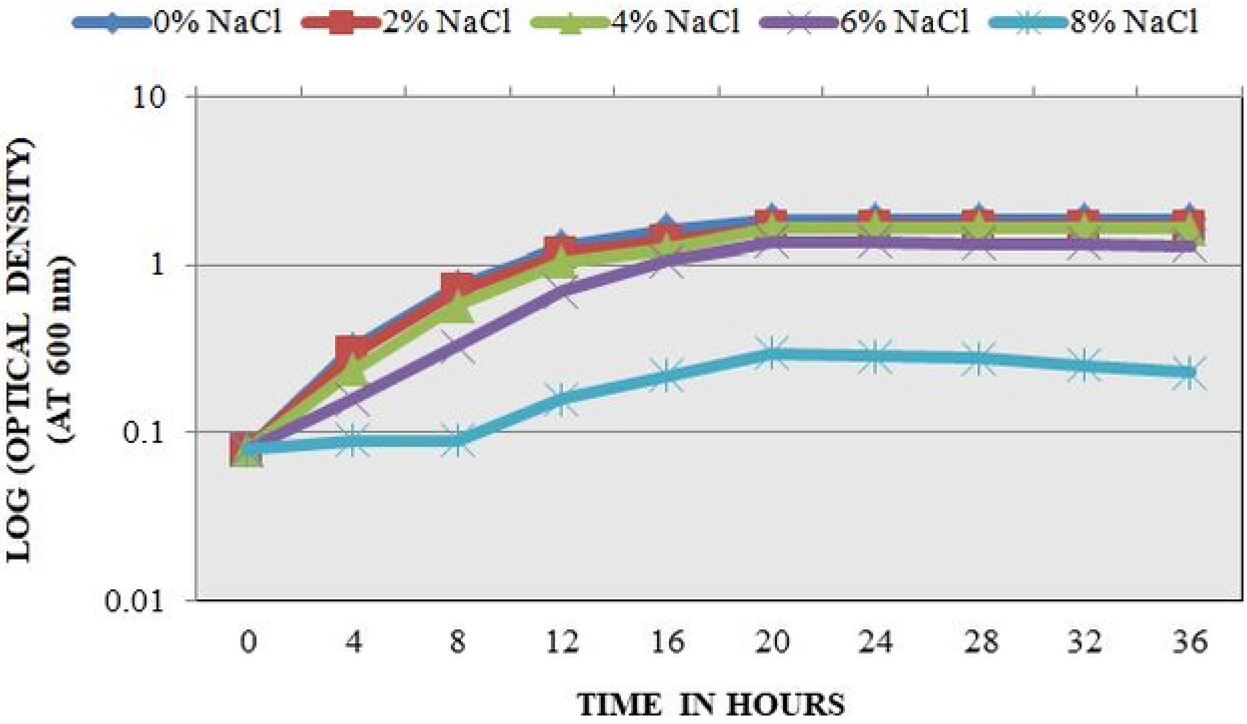

Figure 3. Growth curve of isolate KA31 under different salt concentrations

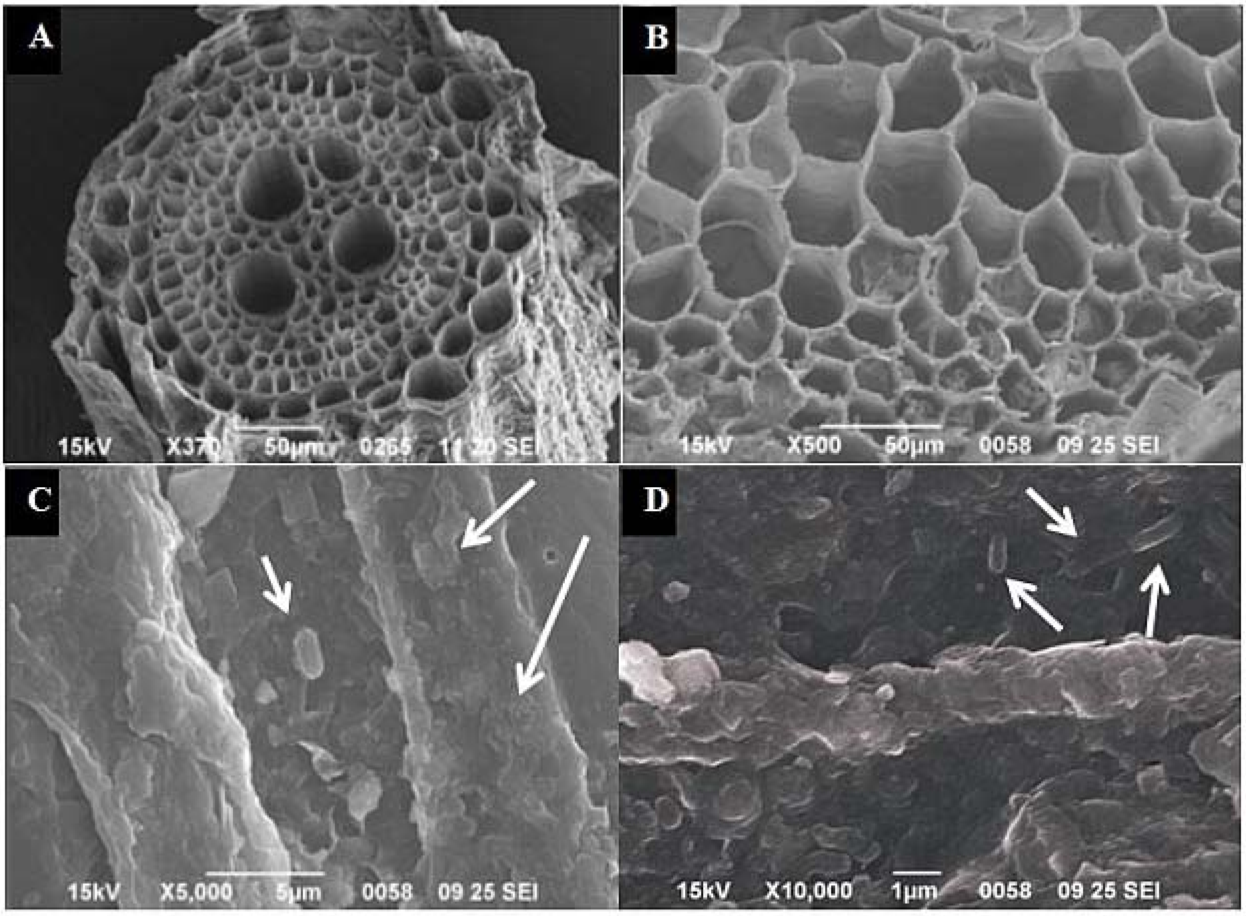

Figure 4. Scanning electron micrographs of wheat roots inoculated by *A. faecalis* KA31. Low magnification view (370x) showing overview of the wheat root (A). Micrographs at higher magnification (500x, 5,000x, 10,000x) showing endophytic bacterial colonization of KA31 in the intercellular spaces as indicated by arrow (B,C,D)

Determining the colonization ability of selected isolate

The selected isolate was tested for plant infectivity assay and it was found that KA31 was able to effectively colonize and reinfect the roots of the host plant i.e., wheat (Fig. 4). Bacteria colonized the plants through cracks formed at the emergence of lateral roots or the zone of root differentiation. No evidence of rhizospheric or endophytic colonization was found in experimental control which rules out the possibility of cross contamination.

Pot experiment

Isolate KA31 was evaluated for its ability to improve growth and yield of wheat in both sterile and non-sterilized soil through pot study. The soil used in pot culture experiments was found to be saline with EC 8.72 dS/m and pH of 8.0. Further the soil properties were: organic carbon content 4.1 g/kg, available nitrogen 0.13 g/kg, available phosphorous 42.1 mg/kg, available K 152 mg/kg, microbial biomass 101.9 mg/kg, and soil organic matter 5.3 g/kg. A significant increase ($P < 0.005$ as detected from Student's t-test) in all the plant growth attributes were evident in plants treated with *A. faecalis* KA31 over the experimental control under saline conditions (Table 2). Increment in root length (129.9%), shoot length (106%), fresh weight (79.85%), dry weight (10.5.6%), and spike length (52.74%) was reported for plants inoculated with KA31 in respect to control. A similar trend of growth promotion was also found in the harvested plants grown in non-sterilized soil conditions.

DISCUSSION

Salinity is one of the major abiotic stress factor inducing several metabolic changes in plants and the major mechanisms involve reduced water absorption, altered nutrient uptake and metabolism, reduction in chlorophyll content, specific ion toxicity, and increase in the production of reactive oxygen species (ROS) (Ashraf et al. 2009). Salt stress causes nutrient disorders in plants and also affects different steps of N metabolism due to altered uptake of N, NO_3^- reduction, NH_4^+ assimilation, decreased amino acid synthesis, and increased activity of hydrolyzing enzymes (Debouba et al. 2007, de Souza et al. 2016). Microbes-mediated salinity tolerance in plants is one of the well-known established phenomena that provide an economically feasible strategy to combat salt stress globally (Jaemsaeng et al. 2018, Kruasuwan and Thamchaipenet 2018).

Plant growth promoting endophytes (PGPE) are essential determinant to biotic as well as abiotic stresses in plants and are considered better in stimulating plant growth compared to rhizospheric bacteria because of their ability to colonize plant interiors. Since endophytes interact closer to plants than rhizosphere and phyllosphere bacteria, they face lesser competition from other microbes and their effects to the plants may be direct and intense (Yadav and Yadav 2017, Afzal et al. 2019). Numerous reports are available on the favorable role of endophytes in plant metabolism (Khan and Doty 2009, Xing et al. 2011, Vigani et al. 2019); however, the role of bacterial endophytes under saline conditions has not

Table 2. Effect of endophytic bacteria KA31 on growth parameters of wheat under saline conditions (pot study)

Treatments	Root length (cm)		Shoot length (cm)		Fresh weight (g)		Dry weight (g)		Tiller no.		Spike length (cm)		No. of grains per spike		Germination (%)	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Control	4.81± 0.11^a	2.83± 0.41^a	15.65± 0.431^a	14.36± 0.35^a	10.92± 0.80^a	8.93± 0.30^a	2.13± 0.31^a	0.88± 0.13^a	2.00± 1.00^a	1.66± 0.57^a	3.83± 0.38^a	3.13± 1.00^a	23.33± 1.5^a	20.00± 1.00^a	73.33	66.66
KA31	11.06± 0.15^b	9.36± 0.56^b	32.32± 0.35^b	28.00± 1.00^b	19.64± 0.89^b	17.00± 0.70^b	4.38± 0.45^b	1.70± 0.40^a	3.33± 1.15^a	2.00± 1.73^a	5.85± 0.29^b	4.05± 0.30^a	32.33± 1.52^b	28.33± 0.577^b	80	76.66

Data is mean ± *SD (n = 3 pots)* of two years. Values with different letters are significantly different by Student's t-test at ($P \leq 0.05$). 1 and 2 are sterilised and non-sterilised, respectively.

been elucidated. PGPE contains multiple plant beneficial traits that help the plant to thrive in unfavourable conditions. In the present study, a salt tolerant bacterial endophyte *A. faecalis* KA31 was isolated from roots of wheat plants and was selected on the basis of nitrogen fixing ability and PGP attributes. The SEM images of inoculated roots exhibited the presence of endophytic association between KA31 and wheat plant. Panigrahi et al. (2020) characterized an endophytic bacterium *Enterobacter cloacae* OS03 for different plant growth promoting capabilities and it was found that the strain significantly enhanced the growth of four crop plant viz. rice, groundnut, black gram, and toria. Improvement in plant growth and enhanced tolerance during a stressful environment was also observed in wheat plants inoculated by two rhizobacterial strains of *A. faecalis* (SBN01 and SBN02) as reported by Babar et al. (2021). Ray et al. (2016) also demonstrated the potential of three endophytic bacterial isolates as biofertilizers and bioprotectants. The isolates displayed high similarity to *Alcaligenes* sp.

N is a vital macronutrient essential for plant growth (Liu et al. 2017) and biological nitrogen fixation is a potential process that maintains the status of N and fertility of soil (Gourion et al. 2015). *A. faecalis* KA31 showed nitrogen fixation capability by growing on nitrogen free medium and the isolate also reduced acetylene to ethylene showing ARA activity. The presence of *nifH* gene amplified by PCR also indicated that the isolate have the ability to fix nitrogen. The nitrogen fixation genes are found in several phylogenetic groups; however amongst them *nifH* is one of the oldest and most functional gene that encodes the Fe-nitrogenase subunit of nitrogenase complex (Rosado et al. 1998, Souza et al. 2013). *nifH* is a useful marker tool to check the N fixing activity (Zehr et al. 2003, Terakado-Tonooka et al. 2008, Jha 2019). Previous studies on *A. faecalis* have primarily focused on its plant growth promoting attributes and demonstrated the bacterium as bioremediator and multifaceted PGPR (Sayyed et al. 2010, Antony et al. 2010, Akintokun et al. 2019). However to the best of our knowledge, there is no report available which depicts the diazotrophic nature of the bacterium. This study illustrates the expression of *nifH* gene and production of ethylene by a novel salt tolerant endophyte *A. faecalis* KA31.

In abiotic stresses, the productivity of wheat is often adversely affected and the yield starts to decline due to low water potential of soil and excess sodium ion accumulation within the plant. It is widely described that soil microorganisms play a pivotal role in stimulating the plant growth and soil health by alleviating the salinity stress (Otlewska et al. 2020, Yan et al. 2015). *Alcaligenes* sp. was reported as an efficient salt tolerant plant growth promoting bacteria by Fatima et al. (2020) and the mechanisms of stress tolerance traits in correlation with salinity gradients were also explained through statistical models. Endophytic bacteria *B. subtilis* and *M. cicero* colonized root tissues of chickpea and were found to be effective in plant growth promotion under saline soil conditions (Egamberdieva et al. 2017). In the current study, the isolate KA31 was examined for NaCl tolerance capacity and the endophytic isolate showed salt tolerance upto 8% NaCl. The application of endophyte to check its impact on growth of wheat showed that KA31 not only increased the growth parameters of wheat but also helped the plants to tolerate saline environments, compared with the un-inoculated ones. Plant growth attributes such as root and shoot length, plant fresh and dry weight, spike length, tiller numbers, and number of grains per spike were remarkably higher in the endophyte-associated plants.

The present study highlights the role of multifaceted endophytic associations in survival and growth of wheat under saline conditions. Endophytic bacterial communities are needed to be explored for their active potential in biological nitrogen fixation; and in this regard the present study reports a novel salt tolerant diazotrophic endophytic isolate of *A. faecalis* KA31, suggesting the presence of active N_2 fixing biosynthetic pathway in strains of *Alcaligenes*.

CONCLUSION

It can be concluded from the study that endophytic association of *A. faecalis* KA31 has significantly ameliorated the negative effects of salinity stress and stimulated the growth of wheat plants. Multifarious PGP attributes including IAA production, siderophore production, solubilization of Zn, P as well as nitrogen fixation activity shown by KA31 might be important factors responsible for the growth promotion of wheat under saline conditions. Application of such novel PGPE renders them to be promising candidates in futuristic bioinoculants and therefore understanding such complex plant-microbe relationships can help in improving the quality and productivity of agricultural crops in the near future.

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