Amelioration of soybean plant from saline-induced condition by exopolysaccharide producing *Pseudomonas*-mediated expression of high affinity K^+-transporter (*HKT1*) gene

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There is a consensus that soil salinization causes decreased agricultural production. Several plants may adapt to survive under high salt stress wherein glycophytes fail to grow. Among transporters in plants, the sodium transporter, also known as high-affinity K^+-transporter (*HKT1*) that comes under the *HKT* gene family, is involved in uptake of sodium through the roots and its recirculation from shoot to root. In the present study, we have examined the role of transporter *HKT1* in soybean plants upon addition of 200 mM NaCl and treatment with *Pseudomonas* sp. strain AK-1. It is reported that *HKT1* is permeable to K^+/Na^+ and systemically alleviates salinity stress through upregulation of gene expression in shoots and down-regulation in roots. Higher transcription levels in shoot recirculate Na^+ from shoot xylem to root phloem whereas lower transcription levels in root do not allow sodium to enter the plants through root cells. We have also examined role of exopolysaccharide (EPS) produced by strain AK-1 which helps in the binding of free Na^+ from soil and thus makes Na^+ unavailable to the soybean plants. Strain with EPS showed decrease in electrical conductivity of soil from 1.1 to 0.9 dS/m in the presence of 200 mM NaCl. In conclusion, treatment with *Pseudomonas* sp. strain AK-1 exhibits significant rise in shoot/root length, number of lateral roots, shoot/root fresh weight and decreased Na^+/K^+ ratio under salinity stress.

Keywords: Abiotic stress, exopolysaccharide, plant growth-promoting bacteria, sodium transporter, soybean plant.

ACCORDING to the UN, worldwide human population will reach 10 billion by 2050. It is considered as a foremost challenge of the 21st century to provide sufficient food to this increasing population. The greatest challenge for survival of the living beings is to improve plant productivity for an agro-ecosystem in a sustainable manner[1]. The global climatic changes are the major constraints affecting agricultural practices worldwide, as they lead to soil degradation through salinization. Gradual degradation of soil also affects harvestable crops in dry and semi-dry regions because of too little, erratic and poorly distributed annual rainfall[1]. Soil salinization may affect ~50% of worldwide cultivated areas with an annual increase in capacity of 500,000 ha (ref. 2). This causes loss in agricultural production along with deterioration of natural terrestrial resources. In India, 8.4 M ha of land is subjected to salinity and alkalinity, with about 5.5 M ha being waterlogged[1]. To overcome soil salinization and improve health of the environment, land-use policies and planning under land management have been introduced for sustainable agricultural development[2,3].

Soil salinity can be attributed to inorganic ions such as HCO_3^-, Mg^{++}, Ca^{++}, K^+, Na^+, Cl^-, SO_4^{2-} and CO_3^{2-} located in pore water of the soil matrix. Their higher content may cause a rise in the electrical conductivity (EC_e). Soil salinization with EC_e greater than 4 dS/m at 40 mM NaCl affects the survival of salt-sensitive plants (glycophytes)[4]. An increase in EC_e leads to two major stresses – ionic and osmotic. As the salt level rises, osmotic stress adversely affects plant parts in the rhizosphere and causes inhibition of H_2O transportation, cell growth and increase in lateral buds[4]. In plants, ionic stress develops upon induction of toxic levels of Na^+, above the threshold level in leaves, loading to mortality and chlorosis/necrosis of leaf tissue. It also decreases essential cellular metabolic activities, including photosynthesis[5], along with reduced enzyme activities[6]. To overcome such stresses and minimize salt phytotoxicity, plants must take up no more than 3% of the Na^+ (ref. 7).

In recent times, it is demonstrated that inclusion of the high-affinity K^+ transporter (*HKT*) in plants results in the intervention of Na^+ transport in plants, especially in rice[8], barley[9], wheat[10] and *Arabidopsis thaliana*[11]. In a heterologous system which includes a single-cell fungus,

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yeast and *Xenopus laevis* oocytes, *HKT1* has been reported to lead Na^+–K^+ transport in cohort and allow Na^+ transport at detrimental millimolar concentration of Na^+ (ref. 11). In addition, mutated *Arabidopsis* plants (*AtHKT1;1*) exhibited an exclusion of Na^+ whereby leads K^+ homeostasis in leaf tissues during salinity stress[12,13]. This selective transport facilitates shoot to root recirculation of Na^+ and also decreases uptake of Na^+ from the root[12,13]. Recently, Chen *et al.*[14] have found that a gene *GmHKT1;4* in soybean regulates the Na^+/K^+ ratio in root tissues and leads to salt tolerance in genetically engineered plants.

It has been reported that plant growth promoting bacteria (PGPB) colonize plant roots and ameliorate growth *in vitro* and *in situ*. Upon induction of stress plants metabolic processes are hindered, which can be conquered by application of PGPB. The PGPB allow growth of plants upon interaction in the spermosphere and rhizopshere through hormonal and nutritional homeostasis, nutrient solubilization and induce tolerance and resistance under abiotic and biotic stresses[15]. Amelioration of salinity stress in plants by employing PGPB has been described in various crops[16–23]. Under salt stress, PGPB help plants to survive by secreting 1-aminocyclopropane-1-carboxylate deaminase (ACC-D) that decreases the level of plant stress hormone C_2H_4. Production of PGPB-mediated exopolysaccharide (EPS) normalizes harmful effect of Na^+ by chelating Na^+ along with other cations, thus ameliorating plant growth in the presence of high level of Na^+ in the soil. In addition to EPS, production of antioxidant enzymes and action of *HKT1* transporter also control Na^+ import in roots, thereby reducing Na^+/K^+ ratio in plant tissues differentially[24].

Until now no report has been published on induced systemic tolerance (IST) in bacterially treated soybean plants for expression of the *HKT1* gene. In the present study, efficacy of *Pseudomonas* strain AK-1 has been examined as PGPB in removing the deleterious effects of salt stress in comparison to non-inoculated and salt-untreated soybean plants. *Pseudomonas* strain AK-1 is reported to display PGP attributes, viz. indole-3-acetic acid (IAA) generation, siderophore, Pi-solubilization and ACC-D activity in 500 mM salt stress and involves stress-responsive enzymes[25].

Materials and methods

Bacterial suspension and treatment

To show PGP characteristics, *Pseudomonas* sp. strain AK-1 (NCBI accession no. KJ511869 and MTCC number 12058) was employed as inoculum. The subcultures were maintained on King's medium B agar (HiMedia, India) at 28° ± 2°C in BOD incubator treated for 24–48 h. Further, inoculation of bacterial culture was deployed on 7-day-old plants, wherein roots were dipped and further incubated in bacterial suspension of concentration 1×10^8 CFU/ml with 0.85% NaCl for 1 h before planting[26]. Control plants roots were treated in sterile water for 1 h.

Soybean variety and seed inoculation

The soybean [*Glycine max* (L.) Merrill] variety JS 9560 was employed. The seeds were treated with 0.1% $HgCl_2$ and 70% ethanol successively for 3 min and repeatedly washed with sterile water (Millipore, Germany). Upon sterilization, seeds were sown in 200 ml propylene cups containing autoclaved soil (treated for three successive days in muslin cloth). In every cup, two seeds were placed at a distance of 15 mm from the edge of the cup. Further, seed-amended cups were placed in a plant growth cabinet (Weiber, ACMAS, India) under optimum conditions (26°C and 16 h/8 h light/dark photoperiod with 70%–80% humidity). After seven days, well-grown plants of equal height were selected for bacterial treatment.

Salt stress treatment

For salt treatment, 200 mM NaCl solution prepared in ½ strength MS medium (Himedia, India) was incorporated in every cup three days in a row devoid of H_2O in case of the 7-day-old treated plantlets. The experiment was conducted by employing randomized block design in the presence of bacterial strain amended with salt and non-salt MS control. To check reproducibility, experiments were performed three times with five replications per treatment. Samples of leaves and roots were collected for RNA isolation, and quantification of Na^+–K^+ ratio in the plants after 15 days of treatment. The treated plants were also observed for lateral roots, root and shoot dry weight along with shoot and root length. Four types of treatments were developed for the study: T1 (without salt stress and strain), T2 (with salt stress), T3 (with salt stress and strain) and T4 (with strain).

Measurement of EC

EC of the soil was measured to find the salt level by employing 1 : 5 soil : water ratio treated with 1 h in shaker sing electrical conductometer.

Quantification of EPS production

EPS was extracted using the protocol of De Vuyst *et al.*[27]. Flasks containing 0–500 mM NaCl in 100 ml nutrient broth were inoculated with 1 ml of 24 h freshly grown bacterial culture with 0.2 OD (1×10^8 CFU/ml) and kept

in an orbital shaker at 150 rpm. Cultures were incubated for 72 h at 28°C. They were centrifuged at 10,000 rpm at 4°C for 15 min and subsequently precipitated with three volumes of pre-chilled acetone. Upon treatment and after 48 h, EPS was recovered by employing centrifugation at 15,000 rpm at 4°C for 20 min and the pellets were heat-dried at 58°C for 24 h in an oven. Weight of the dried pellet was recorded and EPS was quantified (as g 100 ml^{-1} culture). Salt untreated broth was considered as control. Experiments were repeated five times.

Quantification of adsorbed Na^+

The sodium ions adsorbed were calculated using dried pellet of EPS obtained above. For this, extraction was done with HNO_3 for dry powder samples with capacity 0.1 g and then quantified as described by Yuqi *et al.*[28].

Distribution of Na^+/K^+ in plant tissues

The plant tissues (roots and leaves) were washed three times with sterile water (Millipore, Germany) and then the samples were heat-dried at 80°C upon filtration using Whatman filter paper. The obtained dried samples (0.1 g) were then treated with 5 ml 4 M HNO_3 at 37°C and incubated overnight to release the free cations. Samples were further centrifuged at 10,000 *g* for 10 min to recover the supernatant. The resulting supernatant was diluted with sterile water and the ratio of Na^+ and K^+ was determined with the help of an atomic absorption spectroscopy (AAS) (Shimadzu AA-680). Finally, Na^+/K^+ ratio was calculated using the formula: mmol/100 g sterile H_2O (ref. 28).

RNA extraction

After 15 days of treatment, total RNA from plant tissues (roots and leaves) was extracted separately using the protocol of MacRae *et al.*[29], with slight modification. A fresh sample of root and leaf with amount 100 mg was macerated using a mortar and pestle in the presence of liquid N_2 and 1 ml Trizol. Then the recovered macerated liquid suspension was centrifuged at 10,000 rpm at 4°C for 10 min. Organic and inorganic phases were separated by adding 200 µl of chloroform into the supernatant. The solution was thoroughly mixed and incubated at room temperature for 5 min. After mixing, the solution was centrifuged at 12,000 rpm at 4°C for 15 min and the upper aqueous phase was carefully collected. Finally RNA was precipitated by adding 500 µl of isopropyl alcohol Trizol^{-ml} and centrifuged at 12,000 rpm at 4°C for 20 min. The obtained pellet was rinsed twice with 70% alcohol and suspended in 30 µl RNAase-free water. Genomic DNA was removed from samples upon treatment with 2 µl DNAase.

Reverse transcriptase polymerase chain reaction

cDNA synthesis: First-strand cDNA synthesis was performed by employing RT–PCR. For this, reaction mixture was prepared in PCR tube with capacity 6 µl RNA, 1.5 µl oligo $d(T)_{18}$ (1 µg/µl) and 4 µl nuclease-free water. The components were gently mixed for 3–5 sec, incubated at 62°C up to 10 min and then allowed to cool. Further, the tube was placed on ice and the following components were added for 25 µl reaction mixture (including 11.5 µl oligo dT-bound RNA): 1X reaction buffer, 250 µM dNTP, 1.5 mM $MgCl_2$ and 10 U RT. The volume was made up to 25 µl with sterile water (nuclease free). The 25 µl PCR master mix comprised of 5 µl cDNA, 0.6 µM forward and reverse primers, 1.2X reaction buffer, 0.25 µM dNTP and 2.5 µM $MgCl_2$. The following primers were used (5′–3′) for RT–PCR[29]: *Actin*, CGGTGGTTCTATCTTGGCATC (forward)/GTCTTTC-GCTTCAATAACCCTA (reverse) and *HKT1*, TGTCCC-CACCAATGAGAACA (forward)/CAACCAGGAAGC-AACAACGA (reverse). *HKT1* primers were designed using available mRNA coding sequence (CDS) in NCBI. Later, PCR was performed in a thermal cycler at 95°C for 1 min, followed by 35 cycles for 30 sec at 94°C, 56°C for 1 min, 72°C for 90 sec and a final extension of 72°C for 7 min. Each sample was assayed three times. Thereafter, the tubes were incubated at 42°C for 60 min. The reaction was terminated upon incubation at 70°C for 10 min and further tubes were laid on chilled ice. The recovered PCR product was determined on 2% agarose gel and further analysed using UV-Tech Gel-doc system.

Results

Pseudomonas sp. strain AK-1-induced IST via HKT1

Soybean plants inoculated with *Pseudomonas* sp. strain AK-1 showed enhanced root and shoot growth with increased counts of lateral roots compared to salt-treated plants. Biomass with shoot and root weight was also observed to be high for plants treated with the bacterial strain. Upon treatment, T3 and T4 plants exhibited significant shoot and root weight compared to salt-treated plants (Table 1 and Figure 1). Further, strain AK-1 showed alleviation of salinity stress through decreasing Na^+/K^+ ratio and maintaining it so that the plants face less Na^+ toxicity compared to those of the uninoculated plants. There was a change in the Na^+/K^+ ratio in shoot and root. A low value of 0.36 mmol/100 g dry wt Na^+/K^+ ratio was found in T3 shoot, whereas T2 plants showed 4.03 mmol/100 g dry wt Na^+/K^+ ratio. Similarly, 0.86 mmol/100 g dry wt Na^+/K^+ ratio was reported for T3 plants

Table 1. Effect of *Pseudomonas* on shoots, roots, lateral root, shoot fresh weight, root fresh weight and Na^+/K^+ ratio of salt-treated soybean plants

Treatment	Shoots (cm)	Roots (cm)	Lateral roots (nos)	Shoot fresh weight (g)	Root fresh weight (g)	Na^+/K^+ ratio in shoot (mmol/ 100 g dry wt)	Na^+/K^+ ratio in root (mmol/ 100 g dry wt)
T1 (without salt stress and strain)	28^b	12.5^b	35^b	0.641^b	0.361^b	0.2^b	0.32^b
T2 (with salt stress)	24^a	11^a	25^a	0.45^a	0.254^a	4.03^d	4.78^d
T3 (with salt stress and strain)	33^c	14^c	47^c	0.750^c	0.449^c	0.36^c	0.86^c
T4 (with strain)	45^d	17^d	56^d	1^d	0.587^d	0.11^a	0.23^a

Values are means of five replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using DMRT at $P < 0.05$.

Table 2. Exopolysaccharide production by bacterial strain

	NaCl concentration (mM)				
	0	200	300	400	500
Fresh weight (g ml^{-100})	0.65 ± 0.049	0.69 ± 0.032	0.82 ± 0.054	0.87 ± 0.043	0.95 ± 0.057
Dry weight (g ml^{-100})	0.32 ± 0.077	0.36 ± 0.052	0.43 ± 0.079	0.48 ± 0.057	0.53 ± 0.044
Na^+ adsorbed (mmol/100 g dry wt)	1.2 ± 0.043	6.53 ± 0.032	9.94 ± 0.021	11.43 ± 0.034	16.53 ± 0.036

Values are presented as mean ± SD; $n = 5$.

Figure 1. *Pseudomonas* sp. strain AK-1 confers salt tolerance of 200 mM NaCl in soybean. T1, NaCl untreated and *Pseudomonas* uninoculated plants. T2, NaCl-treated plants. T3, NaCl-treated and *Pseudomonas* inoculated plants. T4, *Pseudomonas* inoculated plants.

compared to 4.78 mmol/100 g dry wt Na^+/K^+ ratio for T2 roots. Both T1 and T4 plants showed low Na^+/K^+ ratio. The results clearly show that application of PGPB strain results in IST in soybean plants.

EPS and EC_e

The EPS produced binds to Na^+, when the population density is increased in the root zone, which helps in the alleviation of salinity stress[30]. The bacterial strain produced 0.65 g fr wt of EPS in the absence of NaCl, but when NaCl concentration increased to 300 mM, this increased to 0.82 g fr wt. Likewise, dry weight also increased from 0.32 g in 0 mM NaCl to 0.43 g in 300 mM NaCl (Table 2). Similarly, Na^+ adsorption also increased as the NaCl concentration was increased from 0 to 500 mM (Table 2). This increase in EPS can be attributed to survival of bacterial strain under salinity stress, which can reduce the available Na^+ for plant uptake in the soil. It is also reported that EPS closely bind to soil particles, thus avoiding drought stress[30]. EC_e measures the salinity level in the soil. EPS binds with Na^+ and reduces salinity stress in the soil. Treatments T1 and T4 show equal EC_e, while T2 shows the highest EC_e (1.1 dS/m) (Figure 2). In the case of inoculated soybean plants, EC_e decreased to 0.9 dS/m. This may be the result of EPS which binds to Na^+ ions and makes them unavailable to plants.

Tissue-specific gene expression of HKT1

HKT1 is known to control Na^+ import in roots and regulate Na^+ homeostasis. *HKT1* was investigated in different tissues, viz. leaves and roots – it showed different levels of expression. A band of 230 bp of *HKT1* was found at 56°C annealing temperature (Figure 3). No significant difference was observed between levels of expression in salt-stressed leaf and root, but for T3 plants *HKT1* was expressed significantly in leaves and roots. *HKT1* was upregulated in leaves, which describes its role in the uptake of Na^+ from xylem tissue and transferring it to phloem tissue. Less significant change in *HKT1* was observed in roots of bacterial-treated plants as compared to salt-treated plants. Treatments T1 and T4 showed less *HKT1* expression, which can be justified by low Na^+/K^+ ratio in the tissue samples (Table 1).

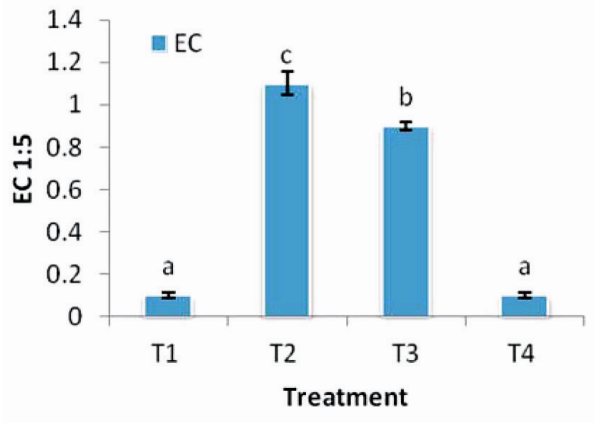

Figure 2. Electrical conductivity (EC) 1 : 5 soil : water ratio under different treatments. T1–T4, same as in Figure 1. Values are presented as mean ± SD; $n = 5$. Different letters in the graph indicate significant differences at $P < 0.05$.

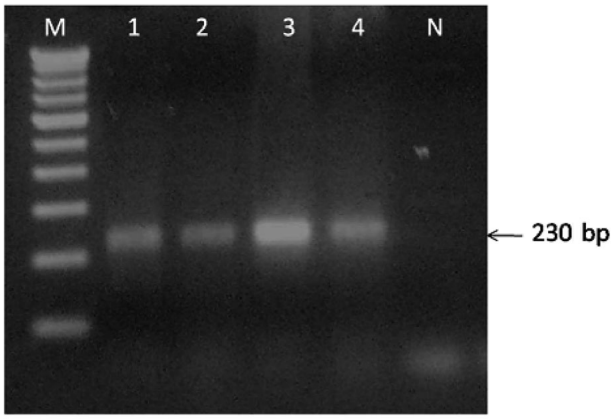

Figure 3. Gel electrophoresis results of reverse transcriptase PCR of *HKT1* gene. Amplicon length of *HKT1* is 230 bp. Also, 2% agarose gel in 1× TAE solution was run for 60 min at 100 V. Lane M, 100 bp DNA ladder (Merk Millipore India). Lane 1, *HKT1* expression in salt-treated shoots; lane 2, *HKT1* expression in salt-treated roots; lane 3, *HKT1* expression in salt + PGPB-treated shoots; lane 4, *HKT1* expression in salt + PGPB-treated roots.

Discussion

In the present study we show that *Pseudomonas* sp. strain AK-1 significantly decreases Na^+/K^+ ratio and maintains Na^+ level in salt-treated plants. The study has been supported by inoculation of strain *Bacillus subtilis* GB03 that imparts tolerance in *A. thaliana* under salt stress[31]. Under salt treatment, *Pseudomonas fluorescens* biotype F and *P. fluorescens* CECT 378^T inoculation in sunflower results an increase in fresh weight of more than 10% followed by 66%, 34% and 16% in leaves, stems and roots respectively, with accumulation of less Na^+/K^+ in plant tissues[32]. The present study also supports tissue-specific regulation of *HKT1* in bacteria-treated shoots. It also shows upregulation in shoots and slight down-regulation in roots compared to salt-treated control, which indicates no change in *HKT1* expression in shoots and roots. In addition, strain AK-1 exhibits increase in PGP characteristics under salt-treated soybean plants. When Na^+ reaches the plant cells through xylem cells, it causes severe damage to plant health. A rise in Na^+ principally increases cytoplasmic Na^+ and leads to changes in Na^+/K^+ ratio (low to high), thereby inhibiting enzyme activities, as most of the enzymes need K^+ in order to function[33]. This altered ratio can be suitably fixed by decreasing Na^+/K^+ ratio using ion transporter *HKT1*. This is the main gene regulating Na^+ and K^+ levels under salinity stress. It has been reported that the *HKT1* transporter belongs to a class of integral proteins (inositol monophosphate) that facilitate cation transport across the plasma membrane of plant cells[34]. Further, *HKT1* was segregated into two subgroups based on their transport selectivity: group 1 was described as Na^+ uniporters, while group 2 allowed both Na^+ and K^+ symport transport under specific conditions, and Na^+ uniport transport at high Na^+ concentration[35]. It has been shown that high levels of Na^+ affect numerous vital cellular processes[35] that are directly correlated with K^+ transport. It is important to consider that K^+ helps in the alleviation of toxic effects of Na^+, thereby leading to high K^+/Na^+ ratio in shoots, and especially in the leaves, showed by salt-sensitive plants (glycophytes)[36].

Recent studies have highlighted the role of *HKT* transporters in salt tolerance[31]. Under salt stress of 100 mM NaCl, *B. subtilis* strain GB03 concurrently down- and up-regulates *HKT1* expression in roots and shoots respectively, thus lowering Na^+ accumulation in *A. thaliana* plants compared with control[31]. Measurement of Na^+ flux[22] and assays for ion accumulation in *HKT1;1* mutant have verified that *AtHKT;1* contributes in the control of both root accumulation and retrieval of Na^+ from the xylem, but devoid of root influx or recirculation in the phloem[37]. Shoot Na^+ exclusion through expression of *HKT1;1* specifically in the mature root stele of *A. thaliana* decreases Na^+ accumulation in the shoot by 37%–64%, which leads to a reduction of Na^+ root-to-shoot transfer. In contrast, plants constitutively expressing *HKT1;1* accumulated high shoot Na^+ and grew poorly[38]. Besides, improved salinity tolerance through cell type-specific expression of *AtHKT1;1* was observed in rice[38]. The over expression of Na^+ and K^+ permeable *HvHKT2;1* transporter in both roots and shoots by low K^+ growth conditions, and in shoots by high Na^+ growth conditions leads to increased salt tolerance by reinforcing the salt including behaviour of barley[39]. Whereas Xue *et al.*[40] found Nernstian shift in Na^+ channel transport properties in *Arabidopsis* root stellar cells of *AtHKT1;1. TaHKT1; 5-D* which helps in retrieval of Na^+ from the xylem vessels in the root and has an important role in restricting the transport of Na^+ from the roots to the leaves in bread wheat and maintained a high K^+/Na^+ ratio in the leaves[41].

EPS produced by the bacterial strain increases the water holding capacity of soil, and helps in the chelation

of metal ions that promote plant growth[42,43]. Bacterial strain used in the study was able to produce EPS in considerable amounts and provided the above properties. Due to the anionic nature of the outer membrane, bacterial strain showed metal chelating property, which helps capture nutrients and minerals as well as detoxification from harmful metals[44]. Nadeem *et al.*[15] reported mitigation of salt by EPS-secreting rhizobacteria which bind to Na^+ ions and ultimately decrease free Na^+ in the soil. The decreased Na^+ ions result in decreased EC_e of the soil. In the case of *P. mendocina*, a PGPB strain produced EPS that bind to Na^+, leading to reduction in high level of Na^+ available for plant uptake[45]. The results of the present study also show that EPS secretion by bacterium significantly reduces EC_e of the soil and helps in the amelioration of plant growth.

Conclusion

The present study shows that *Pseudomonas* sp. strain AK-1 alleviate salinity stress by tissue-specific regulation of *HKT1*. Application of the strain efficiently upregulated *HKT1* expression in shoots, whereas it was downregulated in roots in salt-treated soybean plants. Increase in plant growth characteristics was also found in the presence of strain with decreased Na^+/K^+ ratio in plants. Hence, the results favourably support the use of *Pseudomonas* sp. strain AK-1 as PGPB for the salinity-affected soybean plants.

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