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The effect of salinity-resistant biofilm-forming *Azotobacter* spp. on salt tolerance in maize growth

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Abstract

Increasing soil salinity is among the most detrimental threats restricting crop growth and productivity. In recent years, root inoculation with biofilm-forming plant growth-promoting rhizobacteria (PGPR) has been greatly interested in improving abiotic stress tolerance. This study examined the plant growth-promoting and biofilm-forming potential of *Azotobacter* spp. obtained from maize rhizosphere, tested the salinity effect (up to 300 mM NaCl) on biofilm formation and exopolysaccharide (EPS) production, and evaluated their effect on maize growth at different concentrations (0, 50, and 150 mM) of NaCl under greenhouse conditions. The isolates produced different amounts of indole acetic acid (IAA) (from 0.96 to 7.51 µg mL^{-1}) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (from 2.10 to 19.50 µmol α-ketobutyrate mg^{-1} h^{-1}). On average, the highest biofilm formation was found in *A. chroococcum*. Both biofilm formation and EPS production significantly increased ($p < 0.05$) at 150 and 300 mM NaCl in *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4. These strains also significantly increased ($p < 0.05$) root depth by 44.1–55.9%, shoot height by 52.3–58.8%, fresh root weight by 42.8–52.4%, and fresh shoot weight by 44.6–53.6% at 150 mM NaCl compared with the uninoculated salinity-stressed (control) treatment. Among plant growth regulators, higher biofilm formation and EPS production under increasing salinity exhibited better maize growth under salinity stress. Therefore, these respective strains might be promising bioinoculants for alleviating salinity stress in field experiments.

Keywords: biofilm formation, abiotic stress, inoculation, biofertiliser, *Zea mays*.

Introduction

Salinity negatively affects sustainable agriculture worldwide (Srivastava et al., 2019). An important part of the world's agricultural/cropland is salinity-stressed which has been increasing due to improper irrigation and drainage, overuse of chemical fertilisers and pesticides, waterlogging, and seawater seepage (Srivastava et al., 2019). Increasing salinity decreases the growth and yields of a significant number of plants including maize. Maize (*Zea mays* L.) is a moderately salinity-sensitive plant, thus higher salinity causes substantial losses in its global production (Farooq et al., 2015). The yield might be reduced by up to 50–80% depending on species under salinity stress (Morcillo, Manzanera, 2021). Therefore, it is of utmost importance to find solutions for plant tolerance to salinity stress for sustainable agriculture production.

Salinity stress leads to several plants' physiological, biochemical, and metabolic disorders. Increasing salinity induces water and oxidative stress, ionic toxicity, and nutritional imbalance. Under salinity stress, specific ions such as Na^+, K^+, and Cl^- excessively accumulate in soil and plants. Higher levels of such ions change soil properties which disturbs the nutrient balance and plant-microbe interaction in the rhizosphere, eventually reducing crop growth and productivity (Kumar et al., 2021). Excessive accumulation of such ions also hinders the photosynthetic process by decreasing the chlorophyll content, carbon assimilation, and electron transport activity and generates reactive oxygen species which damage proteins, nucleic acids, and cell membrane integrity (Morcillo, Manzanera, 2021). In addition, an increase in NaCl concentration causes osmotic stress by reducing water potential requiring more energy for water and nutrient uptake (Van Oosten et al., 2018).

To cope with such disorders, plants should be protected from the inhibitory effect of salinity. The use/inoculation of plant growth-promoting rhizobacteria (PGPR) helps in plant growth and the alleviation of salinity stress (Srivastava et al., 2019) by regulating nutritional and hormonal imbalance, the production of growth regulators, nutrient solubilisation, and antagonistic potential against pathogens (Kasim et al., 2016). *Azotobacter* species are free-living rhizobacteria with plant growth promotions and have been widely

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utilised as biofertilisers to improve the yield and quality of various crops (Aasfar et al., 2021); this microorganism can contribute to plant growth and development through various mechanisms including N fixation, IAA, siderophore and HCN production, ACC deaminase activity, and phosphate solubilisation.

In addition to growth promotions, this species can also tolerate salinity stress in plants. Previous studies have demonstrated that inoculation with salinity tolerant *Azotobacter* spp. enhances plant growth under saline conditions by diminishing the adverse effect of salinity (Van Oosten et al., 2018; Abdel Latef et al., 2020). However, under natural conditions, the presence of free-living microorganisms might be disturbed by adverse abiotic factors. Therefore, plant-microbe interactions are crucial for plant growth and development under fluctuating environmental conditions (Kumar et al., 2021). Successful interaction between plants and microbes improves the occurrence of beneficial rhizobacteria, soil health, and crop productivity by ameliorating the stresses (Kumar et al., 2021). Therefore, PGPR should be capable of living on plant surfaces to have a beneficial effect.

To deal with changing environmental conditions and extend their survival, PGPR may prefer a biofilm mode of life. The biofilm lifestyle offers great advantages over planktonic counterparts including increased survivability and long-term persistence of microorganisms (Flemming, Wingender, 2010). It was found that about 99% of bacteria reside in biofilms in natural environments (Wu et al., 2019). Biofilms can be briefly described as microbial communities embedded in self-produced exopolymeric substances and attached to biotic/abiotic surfaces. The biofilm matrix (mainly composed of high-molecular-mass exopolysaccharides) acts as a protective barrier against various stresses and maintains a hydrated environment around biofilm-forming cells increasing water-retaining capacity, and mediates cell adhesion to living or non-living surfaces (Flemming, Wingender, 2010). Recent studies reported that rhizobacterial biofilms provide protection from stressful conditions, reduce microbial competition for nutrients and space, and promote plant growth by ameliorating salinity stress (Kasim et al., 2016; Ansari et al., 2019; Morcillo, Manzanera, 2021).

Considering the potential of PGPR in plant performance and their biofilm-forming ability in mitigating several stresses, the present study aimed (1) to determine the growth-promoting and biofilm-forming capacity of *Azotobacter* species isolated from maize rhizosphere, (2) to test the salinity effect on biofilm formation and exopolysaccharide production by high biofilm-forming isolates, and (3) to evaluate the inoculation of the better biofilm-forming strains with high growth promotions on maize growth at different concentrations of NaCl under greenhouse conditions.

Material and methods

Sampling and isolation of Azotobacter spp. Approximately 30 soil samples were taken from maize (*Zea mays* L., 'Agromar MF 714') rhizosphere soils in three different fields (37°09′29.4″ N 38°57′45.7″ E, 37°09′40.5″ N 38°55′38.0″ E, and 37°09′51.7″ N 38°54′20.5″ E) of Şanlıurfa, Turkey in 2018–2019. The soil samples were collected from a depth of 15–20 cm from the surface, and 1 g of soil samples was mixed with 9 mL sterile water. Up to 10^4–10^5 serial dilutions were prepared and then plated on Ashby-sucrose agar. After a 7-day incubation at 28°C, bright and mucoid colonies were selected and transferred onto Ashby-sucrose agar for purification (Jiménez et al., 2011). For the morphological and biochemical characterisation of the isolates, Bergey's (1994) Manual of Determinative Bacteriology was used. The selected isolates were kept at −20°C in a nutrient medium containing 50% glycerol for further experiments.

Molecular characterisation of Azotobacter spp. DNA isolation and PCR assay. For DNA extraction, the 2% CTAB method was used (Çam et al., 2019). To amplify the 16S rRNA gene region, primer pairs fD1 and rD1 were used (Weisburg et al., 1991). The PCR mixture (50 µL) consisted of ~50–80 ng μL^{-1} DNA, Taq DNA polymerase (1.25 U), 10X Taq buffer $(NH_4)2SO_4$, $MgCl_2$ (25mM), 10 µM primer pairs (fD1/rD1) dNTPs (10mM), and ultrapure PCR H_2O. The protocol developed by the enzyme manufacturer (ThermoScientific, #EP0402) was used to amplify the target region in a T100 Thermal Cycler (Bio-Rad). Initial denaturation was conducted at 95°C for 3 min, followed by 34 cycles of 30 s at 95°C, 30 s at 58°C, and 90 s at 72°C; for the final extension, 72°C for 15 min was applied. The quality of amplification products was confirmed under ultraviolet light in a 1% agarose gel.

Phylogenetic analysis. The polymerase chain reaction (PCR) products were sequenced by a 3500XL Genetic Analyzer (MedSanTek, Turkey). The sequences were manually cleaned and edited using the software FinchTV, version 1.4.0, then aligned by the program BioEdit, version 7.0.5.3. To build the phylogenetic tree, the maximum likelihood (ML) and the maximum parsimony (MP) methods, and Bayesian phylogenetic analysis were conducted. Maximum likelihood and maximum parsimony were performed using the software MEGA X, version 10.1.8 with 1,000 replicates (Kumar et al., 2018). For the maximum likelihood analysis, Tamura-Nei (TrN) with the γ-distributed rate variation was chosen as the most appropriate model of nucleotide substitution (Tamura, Nei, 1993). The maximum parsimony analysis was carried out using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 (Nei, Kumar, 2000). For the Bayesian analysis, the Markov chain Monte Carlo method was run using MrBayes, version 3.2.6 (Ronquist, Huelsenbeck, 2003) with four chains for 100,000 generations, sampling every 10 generations. The first 2,500 trees were evaluated as burn-in. The remaining trees obtained in the Newick format were used to construct the tree using MEGA X. Reference sequences were obtained from NCBI-Blast with accession numbers.

Characteristics of Azotobacter spp. Nitrogen (N) fixation. The N-fixing potential of *Azotobacter* spp. was tested by a N-free malate medium described elsewhere (Weselowski et al., 2016). The isolates were streaked onto N-deficient malate agar and incubated at 28°C for 7 days. The colony formation on N-free minimal agar was assessed as positive for N fixation.

Phosphorus (P) solubilisation. The phosphate solubilisation potential of *Azotobacter* isolates was determined in Petri dishes using Pikovskaya's agar (1% glucose, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01%

$MgSO_4 \cdot 7H_2O$, 0.0005% $MnSO_4$, 0.0005% $FeSO_4$, 0.05% yeast extract, 1.5% agar, 0.1% Ca_3HPO_4, and 0.2% calcium phytate). After the incubation on Pikovskaya's agar for 14 days at 30°C, the clear zone formation around colonies was evaluated as positive. The halo zone diameter was measured according to the metric calculations.

Indole acetic acid (IAA). The method used by Chen et al. (2018) was followed for the detection of IAA production. The isolates were incubated in tryptic soy broth treated with tryptophan (200 μg mL^{-1}) for 3 days at 28°C. Tryptic soy broth without tryptophan was used as a control. After centrifugation at 5500 × *g* for 10 min, orthophosphoric acid (0.1 mL) and Solawaski's reagent (4 mL) were added to 1 mL supernatant and then incubated for 30 min in the dark. The amount of IAA was measured at 530 nm and determined based on a standard graph from pure IAA solution.

Siderophore production. To determine siderophore production, chrome azurol S agar-containing plates were used. The colour changes around the halo from blue to orange/yellow showed the ability to produce siderophore (Ahmad et al., 2008).

Hydrogen cyanide (HCN). Azotobacter isolates were streaked on nutrient agar-containing Petri dishes treated with glycine (4.4 g L^{-1}). Whatman filter paper was soaked in the solution prepared with 2% Na_2CO_3 (sodium carbonate) and 0.5% $C_6H_3N_3O_7$ (picric acid) placed on the top of the dish and then incubated at 28°C for 96 h. The presence of red colour was evaluated as HCN-positive (Ahmad et al., 2008).

ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity of *Azotobacter* spp. was determined by measuring the concentration of α-ketobutyrate produced when ACC deaminase enzyme catalyses the degradation of ACC based on the protocol described by Honma and Shimomura (1978). The content of α-ketobutyrate was measured by comparing the absorbance of samples at 540 nm with standard curves prepared with different concentrations (from 0.1 to 1.0 μmol) of α-ketobutyrate solution.

Sodium chloride (NaCl) tolerance. Azotobacter isolates were streaked onto nutrient agar treated with different concentrations of NaCl (final concentrations were 2, 4, 6, and 8 %) and incubated at 28°C for 2 days. The colony formation on agar medium was assessed as salinity-tolerant.

Biofilm assays. A 40 μL (about 1×10^6 CFU mL^{-1}) *Azotobacter* aliquot was inoculated into borosilicate glass tubes containing nutrient broth mixed with autoclaved pond water in a ratio of 3:1 (600 μL, pH 7.5, 0.04% NaCl) and incubated steadily for 48 h at 28°C. The amount of biofilms was measured using a crystal violet staining procedure at 570 nm by a spectrophotometer (Çam, Brinkmeyer, 2020). Based on the optical densities, the degree of biofilm formation was evaluated as 1 < low (L) < 3 < medium (M) < 6 < high (H) < 9.

After determining the biofilm-forming ability of 30 *Azotobacter* isolates, six strains were selected for the pot experiment based on the production of higher amounts of biofilms, multiple PGP properties, and better germination activity. Biofilm formation by these six isolates was then tested at 0, 50, 150, and 300 mM NaCl. The tested concentrations were selected to check the effect of moderate to high salinity on the salinity sensitive maize seedling. The salinity of pond water was ignored. Biofilm experiments were performed under the same conditions unless stated otherwise. Each experiment was conducted with three independent experiments with three replicates.

Exopolysaccharide (EPS) production of the biofilm-forming strains at different concentrations of NaCl was quantified using a modified Congo red binding assay (Kim et al., 2020). 600 μL of the biofilm-formed cultures were vortexed on high and then centrifuged at 14 000 × *g* for 10 min. After the centrifugation, the pellet was re-suspended in 300 μL of 1% tryptone with Congo red (12 μg mL^{-1}) and then incubated at 37°C for 2 h with shaking. After the incubation, the re-suspension was centrifuged at 14 000 × *g* for 10 min. Congo red-containing supernatant was read at 490 nm by a spectrophotometer. Non-inoculated culture medium serves as a control. The absorbances of samples were subtracted from those of the control groups. The results were averaged from three independent experiments with three replicates.

Determination of seed germination activity. The sterilisation of maize seeds was done by soaking them in 1% NaOCl for 1 min, in 90% C_2H_6O for 4 min, and then washed with autoclaved deionised water six times. After the surface sterilisation, the seeds were treated with 20 selected *Azotobacter* isolates (~1×10^7 CFU mL^{-1}) for 3 h. Next, the treated six seeds were incubated on Petri dishes with filter paper moistened with sterile tap water. After an 8-day incubation at 28 ± 2°C in the dark, six strains showing the highest germination activity were selected to be used in the greenhouse experiment.

Greenhouse experiment. Surface sterilised maize seeds were soaked with the respective six *Azotobacter* isolates (~1×10^7 CFU mL^{-1}) for 4 h and then sown at 2–3 cm depth from the surface in polyethylene cups filled with about 3 kg soil (soil texture clay, class c, pH 7.3, EC 1.7 dS m^{-1}, available N 88.6 mg kg^{-1}, Fe 500 mg kg^{-1}, Mg 250 mg kg^{-1}, available P 32.2 mg kg^{-1}). Ten days after sowing, the soil was re-inoculated with the respective strains in nutrient broth and treated with 0, 50, and 150 mM NaCl. The pot experiment was randomly set up in a block design with a daily temperature of 28 ± 2°C under greenhouse conditions with three replicates and five treatments. Non-treated seeds served as a control. The control groups supplemented an equal amount of nutrient broth without bacteria. Maize seedlings were regularly irrigated with tap water as needed. After 50 days of growth, the plants were collected and washed for measurements. Lengths and fresh weights of both maize root and shoot were determined for each replication.

Statistical analysis. The obtained data were evaluated by the software SPSS Statistics, version 24.0 (IBM Inc.). To compare the mean differences between the biofilm formation and pot experiment, Bonferroni *post hoc* and Fisher's LSD tests were employed. In the pot experiment, the effect of the inoculants was compared by Fisher's LSD test at different NaCl concentrations with respect to the control group.

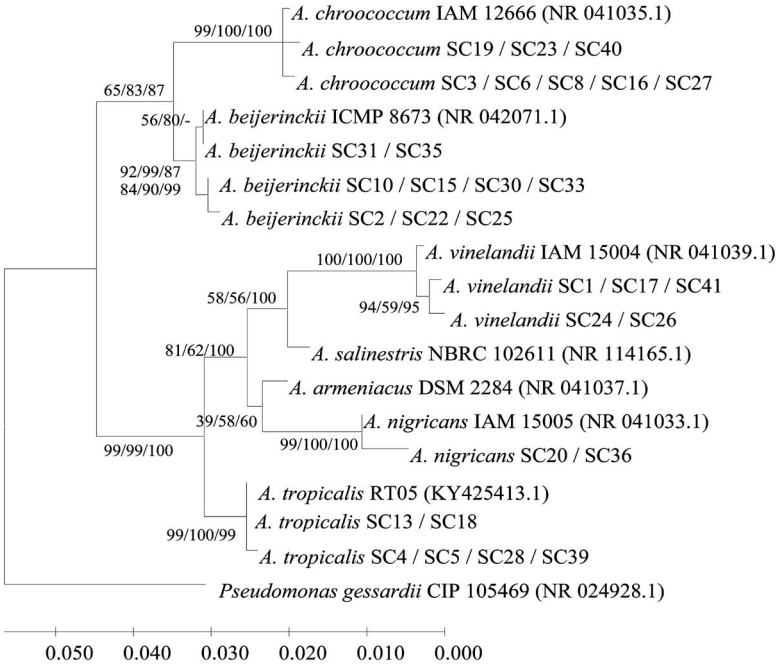
Results

A total of 30 *Azotobacter* isolates were obtained from the maize rhizosphere. As a result of the maximum likelihood, maximum parsimony, and Bayesian analysis, 8 strains were characterised as *A. chroococcum*, 9 as *A. beijerinckii*, 5 as *A. vinelandii*, 2 as *A. nigricans*, and 6 as *A. tropicalis* (Figure 1). The accession numbers are

Table 1. Plant growth-promoting traits of *Azotobacter* spp. isolated from maize rhizosphere

Species	Strain	Accession Number	N fixation	P solubility mm	IAA μg mL^{-1}	Siderophore
A. chroococcum	SC19	ON261573	+	−	4.08 ± 0.24	+
A. chroococcum	SC23	ON261574	+	−	3.04 ± 0.18	+
A. chroococcum	SC40	ON261576	+	−	2.11 ± 0.26	+
A. chroococcum	SC3	ON261569	+	−	3.16 ± 0.43	+
A. chroococcum	SC6	ON261570	+	−	2.65 ± 0.52	+
A. chroococcum	SC8	ON261571	+	2	0.96 ± 0.07	+
A. chroococcum	SC16	ON261572	+	3	2.04 ± 0.15	+
A. chroococcum	SC27	ON261575	+	−	1.97 ± 0.21	+
A. beijerinckii	SC31	ON261583	−	−	2.15 ± 0.09	−
A. beijerinckii	SC35	ON261585	+	−	6.24 ± 0.47	−
A. beijerinckii	SC10	ON261578	+	−	4.20 ± 0.22	+
A. beijerinckii	SC15	ON261579	+	−	1.22 ± 0.14	+
A. beijerinckii	SC30	ON261582	+	−	2.35 ± 0.52	+
A. beijerinckii	SC33	ON261584	+	−	5.47 ± 0.31	−
A. beijerinckii	SC2	ON261577	+	−	5.09 ± 0.73	−
A. beijerinckii	SC22	ON261580	+	1	1.16 ± 0.05	+
A. beijerinckii	SC25	ON261581	+	−	1.62 ± 0.26	+
A. vinelandii	SC1	ON261586	+	1	4.22 ± 0.81	−
A. vinelandii	SC17	ON261587	+	−	2.65 ± 0.48	+
A. vinelandii	SC41	ON261590	+	−	2.15 ± 0.05	−
A. vinelandii	SC24	ON261588	+	−	2.25 ± 0.18	+
A. vinelandii	SC26	ON261589	+	−	4.33 ± 0.41	−
A. nigricans	SC20	ON261591	+	−	3.22 ± 0.29	+
A. nigricans	SC36	ON261592	+	−	5.28 ± 0.33	+
A. tropicalis	SC13	ON261595	+	−	1.98 ± 0.82	+
A. tropicalis	SC18	ON261596	+	−	3.60 ± 0.94	+
A. tropicalis	SC4	ON261593	+	−	7.51 ± 0.76	−
A. tropicalis	SC5	ON261594	+	−	3.28 ± 0.03	+
A. tropicalis	SC28	ON261597	+	−	1.16 ± 0.14	−
A. tropicalis	SC39	ON261598	+	−	4.01 ± 0.12	+

"+" and "−" indicate "positive" and "negative", respectively



Note. Numbers on the nodes demonstrate the bootstrap values of the maximum likelihood / maximum parsimony / Bayesian analysis, respectively.

Figure 1. Phylogenetic tree of *Azotobacter* species isolated from maize rhizosphere

given in Table 1.

All isolates obtained showed N-fixing ability, except the strain SC31. Of all *Azotobacter* isolates, phosphate solubilising activity was found in strains *A. chroococcum* SC8 and SC16, *A. beijerinckii* SC22, and *A. vinelandii* SC1. The highest P solubilisation was seen in *A. chroococcum* SC16 with a 3 mm diameter. *Azotobacter* isolates tested produced different amounts of IAA (from 0.96 to 7.51 μg mL^{-1}). The highest amount of IAA was recorded as 7.51 μg mL^{-1} in *A. tropicalis* SC4

(Table 1).

Of the 30 isolates tested, 21 strains (70%) produced siderophore. The highest rate of siderophore production was in *A. chroococcum* strains (100%), HCN production was found in 23 isolates (77%), and ACC deaminase activity was detected in all the isolates tested. The content of ACC deaminase ranged from 2.10 to 19.50 µmol α-ketobutyrate mg^{-1} h^{-1}. The highest content of ACC deaminase was found in *A. beijerinckii* SC2

Table 2. Characteristics of *Azotobacter* spp. isolated from maize rhizosphere

Strain	HCN	ACC deaminase µmol α-ketobutyrate mg^{-1} h^{-1}	NaCl concentration				Biofilm formation
			2%	4%	6%	8%	
SC19	+	11.80 ± 2.72	+	+	+	+	H
SC23	+	11.61 ± 1.16	+	−	−	−	M
SC40	+	11.08 ± 0.75	+	−	−	−	H
SC3	+	8.31 ± 1.42	+	−	−	−	M
SC6	+	12.65 ± 2.07	+	−	−	−	H
SC8	+	10.16 ± 0.49	+	+	−	−	H
SC16	+	12.87 ± 1.14	−	−	−	−	H
SC27	−	9.75 ± 1.51	+	−	−	−	M
SC31	−	6.24 ± 0.24	+	+	+	−	L
SC35	−	4.72 ± 0.76	+	+	−	−	L
SC10	+	13.01 ± 2.01	+	+	−	−	H
SC15	+	2.22 ± 0.19	+	−	−	−	M
SC30	+	4.11 ± 1.03	+	+	+	−	L
SC33	+	5.12 ± 1.11	+	+	+	+	L
SC2	+	19.50 ± 2.76	+	−	−	−	M
SC22	+	10.65 ± 2.23	−	−	−	−	H
SC25	+	14.23 ± 3.08	+	−	−	−	L
SC1	−	11.08 ± 0.85	+	−	−	−	H
SC17	−	11.65 ± 1.22	−	−	−	−	L
SC41	+	3.66 ± 0.46	+	−	−	−	L
SC24	+	11.58 ± 2.17	+	−	−	−	M
SC26	+	10.11 ± 1.18	+	+	−	−	H
SC20	+	11.72 ± 2.07	−	−	−	−	M
SC36	−	4.68 ± 0.14	+	+	−	−	L
SC13	+	11.77 ± 1.35	+	+	+	−	H
SC18	+	17.22 ± 1.05	−	−	−	−	L
SC4	+	6.17 ± 0.83	+	+	+	+	H
SC5	+	6.27 ± 0.17	+	+	−	−	L
SC28	−	8.10 ± 1.02	+	+	−	−	M
SC39	+	2.10 ± 0.09	+	+	−	−	M

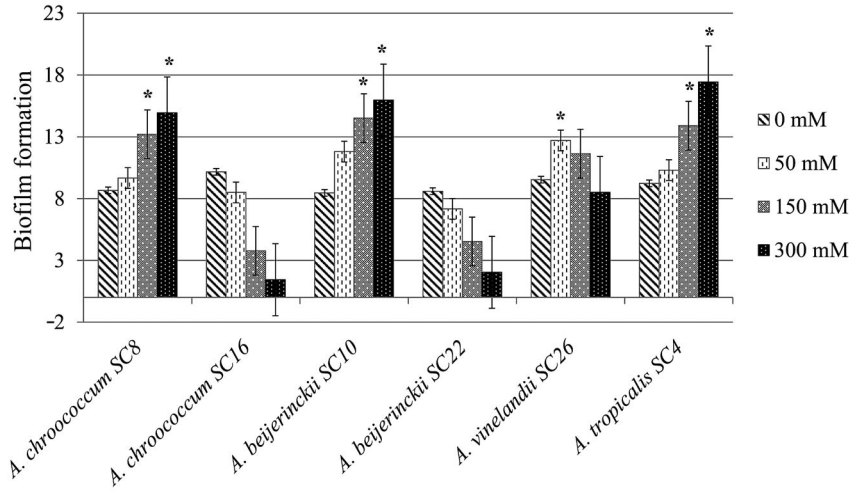
Note. Optical density (OD) at 570 nm; 1 < low (L) < 3 < medium (M) < 6 < high (H) < 9 at 28°C; "+" and "−" indicate "positive" and "negative", respectively.

(Table 2).

Salinity-resistance ability of the isolates was tested at different concentrations of NaCl (Table 2). *Azotobacter* spp. demonstrated various degrees of resistance to different NaCl concentrations. Of 30 isolates, 25 (83%) could grow at 2% NaCl, almost half (~47%) of the isolates were resistant to 4% NaCl, and only strains *A. chroococcum* SC19, *A. beijerinckii* SC33, and *A. tropicalis* SC4 showed tolerance up to 8% NaCl. The biofilm forming ability of *Azotobacter* spp. was tested at 28°C with no salt supplementation. All the strains produced different amounts of biofilms (Table 2).

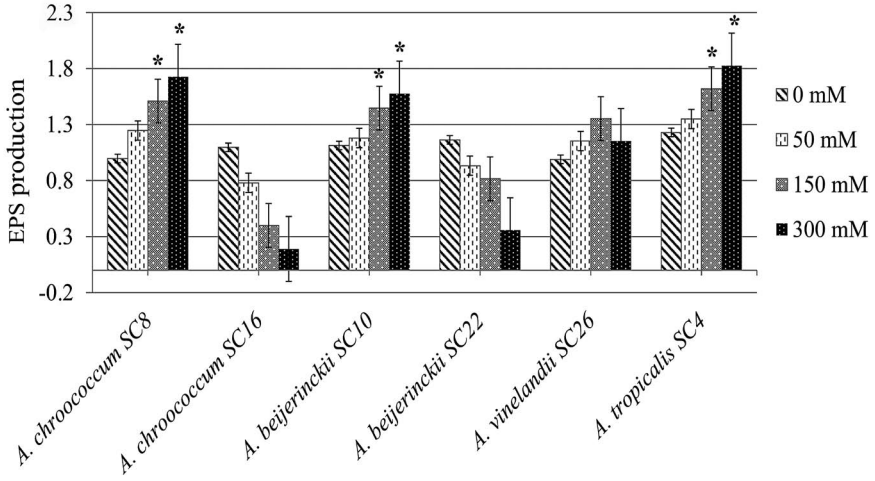
The biofilm formation and EPS production of six strains selected for the pot experiment were tested at different NaCl concentrations. Both biofilm formation and EPS production significantly increased (Bonferroni *post hoc* test, $p < 0.05$) at 150 and 300 mM NaCl in strains *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4 (Figures 2 and 3). These three strains were resistant to up 300 mM NaCl (data not shown). Of the strains tested, the biofilm formation and EPS production by *A. chroococcum* SC16 and *A. beijerinckii* SC22 decreased with increasing salinity. Furthermore, a strong correlation ($p < 0.05$) was found between the biofilm formation and EPS production in the strains tested.

The application of all *Azotobacter* isolates selected for the pot experiment significantly increased (Fisher's LSD test, $p < 0.05$) plant growth parameters tested with no NaCl addition, except *A. beijerinckii* SC22. Increasing NaCl concentration considerably reduced the length and weight of roots and shoots of the inoculated plants and the uninoculated (control) group. The maximum reduction was found at 150 mM NaCl in all the growth parameters regardless of inoculation/non-inoculation. The minimum reduction in the growth parameters was recorded in the maize seedlings treated with strains *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4 under higher salinity stress. These strains significantly increased (Fisher's LSD test, $p < 0.05$) maize growth parameters in the presence of either 50 or 150 mM NaCl compared to the non-treated control. The respective strains promoted root depth by 44.1–55.9%, shoot height by 52.3–58.8%, fresh root weight by 42.8–52.4%, and fresh shoot weight by 44.6–53.6% at 150 mM NaCl compared with the uninoculated treatment (Table 3).



Note. Bonferroni *post hoc* test; mean ± SE; $p < 0.05$; * – difference significant compared to 0 mM NaCl.

Figure 2. Biofilm formation by *Azotobacter* isolates at different concentrations of NaCl



Note. Bonferroni *post hoc* test; mean ± SE; $p < 0.05$; * – difference significant compared to 0 mM NaCl.

Figure 3. Exopolysaccharide (EPS) production by *Azotobacter* isolates at different concentrations of NaCl

Table 3. Effect of *Azotobacter* spp. inoculation on maize growth at different salinity levels

	Root depth cm			Shoot height cm		
	0 mM	50 mM	150 mM	0 mM	50 mM	150 mM
Control	22.3 ± 3.05	18.6 ± 2.08	14.3 ± 1.53	45.6 ± 5.69	38.6 ± 4.04	30.6 ± 1.53
SC8	30.6 ± 3.51*	28.6 ± 3.79*	22.3 ± 4.04*	58.3 ± 4.04*	54.6 ± 7.09*	48.3 ± 6.11*
SC16	28.6 ± 1.53*	22.3 ± 4.51	17.3 ± 3.51	57.3 ± 6.51*	43.6 ± 5.03	35.6 ± 3.06
SC10	28.3 ± 2.52*	26.6 ± 2.08*	21.3 ± 4.73*	59.3 ± 5.86*	55.3 ± 5.69*	48.6 ± 6.03*
SC22	25.6 ± 3.79	23.3 ± 4.51	16.6 ± 2.52	53.3 ± 6.03	41.6 ± 4.04	31.3 ± 4.51
SC26	28.3 ± 1.53*	21.6 ± 3.21	14.6 ± 2.51	56.3 ± 5.03*	43.6 ± 5.51	33.3 ± 5.51
SC4	29.6 ± 3.05*	27.6 ± 3.05*	20.6 ± 2.52*	57.3 ± 7.64*	52.3 ± 6.51*	46.6 ± 7.09*
	Fresh root weight g			Fresh shoot weight g		
	0 mM	50 mM	150 mM	0 mM	50 mM	150 mM
Control	3.1 ± 0.31	2.7 ± 0.31	2.1 ± 0.30	9.6 ± 1.37	7.8 ± 0.78	5.6 ± 0.49
SC8	3.9 ± 0.36*	3.7 ± 0.41*	3.0 ± 0.38*	13.5 ± 1.87*	11.5 ± 1.50*	8.6 ± 1.12*
SC16	4.0 ± 0.51*	3.2 ± 0.42	2.2 ± 0.41	12.8 ± 1.18*	8.3 ± 1.01	5.5 ± 0.92
SC10	4.2 ± 0.46*	3.8 ± 0.35*	3.2 ± 0.25*	12.7 ± 1.65*	10.5 ± 1.22*	8.2 ± 1.40*
SC22	3.5 ± 0.59	2.9 ± 0.65	2.1 ± 0.45	10.6 ± 1.68	9.4 ± 1.16	5.4 ± 1.20
SC26	4.0 ± 0.38*	3.1 ± 0.45	2.0 ± 0.51	12.8 ± 2.04*	9.8 ± 1.05	6.3 ± 0.97
SC4	4.1 ± 0.40*	3.6 ± 0.45*	3.1 ± 0.25*	12.6 ± 1.56*	11.3 ± 1.46*	8.1 ± 1.01*

* – difference significant according to Fisher's LSD test compared to the uninoculated (control) group ($p < 0.05$).

Discussion

Salinity is one of the most deleterious external stressors and markedly reduces crop productivity. Therefore, an effective strategy is required to mitigate salinity stress on plants. Inoculation with PGPR enhances crop growth and productivity through multiple PGP activities under stressful conditions (Kasim et al., 2016). However, considering the sensibility of planktonic microorganisms under harsh environmental conditions and the crucial contribution of biofilms together with EPSs in reducing the impact of salinity stress, the application of biofilm-forming and EPS-producing PGPR may be a better alternative to the treatment of free-living PGPR.

In the current study, *Azotobacter* strains collected from maize rhizosphere exhibited different degrees of biofilm formation. Similar to our findings, Altaf and Ahmad (2017) revealed that the biofilm-forming ability of *Azotobacter* spp. varies from strain to strain. In that study, *A. vinelandii* was the most effective biofilm producer, but in our experiment, *A. chroococcum* strains formed higher amounts of biofilms on average compared to other species isolated. This difference might be due to different growth conditions (i.e., culture medium, temperature, incubation time, and substrate type). Strain-to-strain differences might also affect the biofilm-forming ability, as observed in different microorganisms (Çam, Brinkmeyer, 2020; Kim et al., 2020).

The results of current experiment revealed that the biofilm formation by strains *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4 significantly increases with increasing salinity up to 300 mM. This result is in line with earlier studies showing that higher salt concentration promotes biofilm formation by some agriculturally important rhizobacteria (Kasim et al., 2016; Ansari et al., 2019). The increase in EPS production might explain enhanced biofilm formation under increasing salinity. In our experiment, the biofilm formation followed a pattern similar to EPS production. Likewise, in the study of Ansari et al. (2019), at different concentrations of NaCl, biofilms and EPSs were produced in a similar pattern. Qurashi and Sabri (2012) also observed that biofilm formation is favoured by increased EPS production under salinity stress. In the present study, EPS was of great importance to *Azotobacter* biofilms. It is well-known that EPSs are the major component of microbial biofilm matrix and determine the mechanical stability of biofilm architecture, and the mutants with the loss of EPS-encoding genes were unable to form mature biofilms (Flemming, Wingender, 2010) which highlights a significant contribution of EPSs to biofilm formation.

Additionally, *Azotobacter* cells may form biofilms as a protective response to salinity stress. The biofilm formation is considered a survival strategy for protection against stress factors (Çam, Brinkmeyer, 2020). As also reviewed by Morcillo and Manzanera (2021), the biofilm formation contributes to the water-holding capacity of biofilm cells by the presence of EPSs protecting the biofilm-forming cells against salt-induced osmotic stress. This is because EPSs have the potential to maintain a hydrated layer around microbes decreasing water loss which increases the survivability of microorganisms under water-limited conditions (Morcillo, Manzanera, 2021).

Five of the six *Azotobacter* strains selected significantly increased all maize growth parameters tested under pot greenhouse conditions without NaCl supplementation compared to the untreated control. All the strains were HCN-positive and high-biofilm producers. Overall, no consistency was observed between the multifunctional PGP traits of the strains and maize growth. For example, *A. beijerinckii* SC22 with a low IAA production showed phosphate solubilisation and siderophore activity but could not significantly increase plant growth. On the other hand, *A. tropicalis* SC4 with a high IAA production did not exhibit phosphate solubilisation and siderophore activity but significantly increased all growth parameters. *A. chroococcum* SC8 and SC16 with phosphate solubilisation and siderophore activity produced a low amount of IAA but significantly increased maize growth. This inconsistency might be attributed to the action of one/more PGP traits of *Azotobacter* strains on maize growth. Our findings follow a recent study of Akinrinlola et al. (2018) showing that a strain with one physiological trait exhibits higher growth promotion than those with multifarious characteristics or *vice versa*. Akinrinlola et al. (2018) concluded that efficacy/consistency in growth promotion could not be predicted by any physiological traits recommending that effective strains should be selected through pot tests rather than *in vitro* physiological assays.

Increasing salinity decreased maize growth parameters in all treatments. However, the growth parameters of the maize seedlings treated with strains *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4 were still significant compared with the uninoculated salinity-stressed (control) group. PGPR with ACC deaminase activity protects plants against several stress factors including salinity stress by regulating ethylene content in plants (Glick, 2014). A plant hormone ethylene modulates plant growth and metabolism and is also involved in the increased tolerance to salinity stress via its precursor ACC. In our experiment, ethylene content in salinity-stressed maize were not measured, but it was expected that endogenous ethylene content would be expressed in response to NaCl stress and that the strains exhibiting a high ACC deaminase activity would reduce ethylene content.

Conversely, no correlation was observed between the content of ACC deaminase in inoculants and increased maize growth under salinity stress. Only two of five inoculants with a significant content of ACC deaminase activity (over 10 μmol α-ketobutyrate mg^{-1} h^{-1}), A. *chroococcum* SC8 and *A. beijerinckii* SC10, tolerated salinity stress and promoted maize growth. However, *A. tropicalis* SC4 with a comparatively low ACC deaminase activity significantly increased plant growth. In our experiment, ACC deaminase's effect was unclear in *Azotobacter*-inoculated maize under salinity stress. This may be partially due to the contradictory effect of ethylene in salinity responses since ethylene might have positive/negative effects in modulating plant resistance to saline-induced stress (Tao et al., 2015).

The excessive accumulation of Na^+ in response to a high salinity results in osmotic and mineral imbalance, and ion toxicity disturbing plant metabolisms and photosynthetic attributes eventually reducing crop growth and productivity (Morcillo, Manzanera, 2021). It has been documented that *Azotobacter* spp. can confer tolerance to salinity stress. The root inoculation with salt-resistant *Azotobacter* spp. stimulated plant growth

under saline conditions by alleviating salinity stress (Van Oosten et al., 2018; Abdel Latef et al., 2020). However, such free-living microorganisms might be adversely influenced by biotic/abiotic factors in the rhizosphere. Biofilm formation is believed to be an effective survival strategy for microbes in the rhizosphere and during root colonisation (Ansari et al., 2019). Cells in biofilms are well-protected against external stresses due to the protective mechanisms of the EPS matrix (Flemming, Wingender, 2010). The results of current experiment revealed that the three strains: *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4, producing high amounts of biofilms with increasing salinity significantly increased ($p < 0.05$) the growth of salt-stressed maize seedlings compared with the uninoculated treatment. In contrast, no significant increase was found in the stressed maize seedlings treated with low biofilm-forming strains at higher salinity. These findings may explain the importance of biofilm formation under salinity stress conditions. Our findings are consistent with those of the earlier studies (Kasim et al., 2016; Ansari et al., 2019) which demonstrated that the inoculation of effective biofilm producers at higher salt concentrations improves root colonisation and plant growth by alleviating the deleterious effect of salinity stress.

The underlying reason between the biofilm formation and increased maize growth under salinity stress might be the EPSs, the main composition of biofilms. EPSs can chelate cations including Na^+, Ca^{2+}, Mg^{2+}, and K^+ from salts, restricting the accumulation of such ions into plants and maintaining ion homeostasis promoting plant growth through the mitigation of salinity stress (Morcillo, Manzanera, 2021). Kasotia et al. (2016) observed that EPS-producing *Pseudomonas* strains significantly enhance soybean growth under NaCl-treated conditions by decreasing the electrical conductivity of soil from 1.1 to 0.9 dS m^{-1} through Na^+ chelation. In our experiment, the NaCl concentration in plant tissues was not tested, but it was found that *Azotobacter* EPSs might absorb Na^+ from soil making such cation inaccessible to plants consequently lowering Na^+ to K^+ ratio in the tissues and improving maize growth under salinity stress.

In addition to the cation-binding potential, EPSs might stimulate soil aggregation around maize roots. In our samples, more soil aggregation was found around maize roots inoculated by more EPS-producing strains than those producing less EPSs (data not shown). Qurashi and Sabri (2012) found that higher salinity induces EPS production enhancing soil aggregation around plant roots and improving salinity tolerance. Morcillo and Manzanera (2021) suggested that the biofilm formation along with the binding potential of EPSs makes soil particles bound to the roots enhancing plant growth under salinity stress, because the sticky nature of EPSs helps in cell-cell aggregation and is important for bacterial adhesion to the roots. Increasing salinity decreases plants' water and nutrient uptake leading to osmotic stress and interrupting plant metabolisms (Srivastava et al., 2019). However, enhanced root-adhering soils by EPSs improve water and nutrient uptake by roots enhancing soil texture increasing water retention around roots and reducing water loss under osmotic stress; thus contributing to plant growth under saline conditions (Tewari, Arora, 2014; Morcillo, Manzanera, 2021). Aside from all the adverse effect of salinity mentioned above, Na^+ indirectly decreases soil aggregation and stability through reduced plant productivity (Bronick, Lal, 2005). Thus, EPS-producing PGPR may be an ideal candidate for stress tolerance and crop productivity.

Cation chelation and contribution to soil aggregation lie under the sticky nature of EPSs. The binding properties of EPSs depend on their composition and amount. The Na^+-binding potential of EPSs in *Rhodopseudomonas palustris* was attributed to the presence of the polysaccharide largely composed of D-galacturonic acid (Nunkaew et al., 2015). Furthermore, EPS composition is significantly changed at different NaCl concentrations (Fischer et al., 2003; Tewari, Arora, 2014). Increasing salinity changed the sugar composition of EPSs from galactose to different kinds of sugars (i.e., rhamnose or trehalose) which in turn might increase salinity tolerance and water retention of plants (Tewari, Arora, 2014). Likewise, Fischer et al. (2003) revealed that EPS composition is mainly glucose under non-stressed conditions, but at 300 mM NaCl the composition is mainly changed into galactose in *Azospirillum brasilense*. Tewari and Arora (2014) suggested that sugar components function as a carbon source that protects microbes from salinity stress and changes the water potential indicating a strong relationship between EPS production and salinity tolerance. In our experiment, there was an association between the maize growth, biofilm, and EPS production by *Azotobacter* strains at different concentrations of NaCl. The conferred tolerance of maize seedlings to salinity stress may be due to the changed internal components of biofilm EPSs. Future studies need to elucidate the EPS composition of *Azotobacter* biofilms under different salinity.

Conclusions

The present study showed that selective *Azotobacter* strains such as *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* had the potential of producing more biofilm and exopolysaccharide (EPS) and contributed to the plant growth much better than those forming less biofilm and EPS under salinity stress. Therefore, future investigations should focus on the biofilm-forming and EPS-producing potential of plant growth-promoting rhizobacteria (PGPR) bioinoculants and other growth promotions to ameliorate salinity stress.

The biofilm formation and EPS production followed a similar pattern in response to different NaCl concentrations indicating the importance of EPSs in the formation of *Azotobacter* biofilm. Among the PGP traits, the biofilm formation and EPS production had a greater impact on salinity stress tolerance. The root inoculation with strains *A. chroococcum* SC8, *A. beijerinckii* SC10, and *A. tropicalis* SC4 exhibiting more biofilm and EPS production significantly increased the growth of maize seedlings under salinity-stressed conditions compared to the uninoculated (control) groups. In the field experiments, these strains might be applied as efficient bioinoculants on maize seedlings under salinity stress.

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Druskos stresui atsparių bioplėvelę formuojančių *Azotobacter* spp. bakterijų įtaka auginant kukurūzus

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Santrauka

Viena žalingiausių grėsmių, ribojančių pasėlių augimą ir produktyvumą, yra didėjantis dirvožemio druskingumas. Pastaraisiais metais, siekiant padidinti augalų atsparumą abiotiniam stresui, vis labiau domimasi bioplėvelę formuojančiomis ir augalų augimą skatinančiomis rizobakterijomis. Eksperimento metu tirtas iš kukurūzų rizosferos gautų *Azotobacter* spp. bakterijų augalų augimą skatinantis ir bioplėvelę formuojantis potencialas, nustatyta druskos koncentracijos (iki 300 mM NaCl) įtaka bioplėvelės formavimuisi bei egzopolisacharidų (EPS) susidarymui ir įvertinta jų įtaka kukurūzų augimui esant įvairioms (0, 50 ir 150 mM) NaCl koncentracijoms šiltnamio sąlygomis. Izoliatai išskyrė indolo acto rūgštį (IAA) (nuo 0,96 iki 7,51 µg mL^{-1}) ir 1-aminociklopropano-1-karboksilo rūgšties (ACC) deaminazę (nuo 2,10 iki 19,50 µmol α-ketobutirato mg^{-1} h^{-1}). Izoliatai buvo nevienodai atsparūs įvairioms NaCl koncentracijoms ir suformavo skirtingas bioplėveles. Vidutiniškai daugiausia bioplėvelės suformavo *A. chroococcum*. Esant 150 ir 300 mM NaCl koncentracijoms, *A. chroococcum* SC8, *A. beijerinckii* SC10 ir *A. tropicalis* SC4 kamienai reikšmingai padidino ($p < 0,05$) ir bioplėvelės formavimąsi, ir EPS susidarymą. Esant 150 mM NaCl koncentracijai, šie kamienai taip pat reikšmingai padidino ($p < 0,05$) šaknų ilgį (44,1–55,9%), daigų aukštį (52,3–58,8%), naujų šaknų svorį (42,8–52,4%) ir naujų daigų svorį (44,6–53,6%), palyginti su neinokuliuota druskos streso paveikta (kontroline) grupe. Kuo didėjant druskingumui augalų augimo reguliatoriai daugiau suformavo bioplėvelės ir pagamino EPS, tuo kukurūzai geriau augo druskos streso sąlygomis. Taigi, šie kamienai gali būti perspektyvūs bioinokuliantai, mažinantys dirvožemio druskingumo stresą lauko eksperimentuose.

Reikšminiai žodžiai: bioplėvelės formavimas, abiotinis stresas, inokuliavimas, biotrąšos, *Zea mays*.