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RESEARCH

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Mechanisms involved in plant growth promotion by *Enterobacter cloacae* DJ under salinity-alkalinity stress

Yajun Fan[1], Hanna Wang[1], Zhe Zhang[1], YuXing Li[1], Ziyi Zhao[1] and Xiuzhen Ni[1*]

Abstract

Background Plant-promoting bacteria are safer alternatives to pesticides and fertilizers, reduce environmental pollution, and increase crop yields. We isolated an *Enterobacter cloacae* strain DJ with plant-promoting effects from the rhizosphere soil of a plant (*Leymus chinensis* (Trin.) Tzvel) in the western region of Jilin Province of China and investigated the mechanisms underlying the adaptation of the DJ bacteria to salinity-alkalinity environments and the molecular mechanisms of the cross-talk between DJ bacteria and cucumber seedlings.

Results The average diameter of the colonies on the salinity-alkalinity medium after incubation for 24 h was 3.3 cm, and this was significantly higher than the 1.9 cm diameter in ADF medium ($p < 0.01$). Comparative proteomic analysis revealed that 188 differentially expressed proteins, comprising 116 upregulated and 72 downregulated proteins, significantly changed in salinity-alkalinity groups compared to the control groups. The top one upregulated pathway of KEGG enrichment was bacterial chemotaxis, DJ bacteria adapted to salinity-alkalinity environments by upregulating the genes associated with bacterial chemotaxis. The contents of putrescine in salinity-alkalinity and control groups were 4.73 μg/mL and 3.02 μg/mL, whereas the contents of spermidine were 46.18 ng/mL and 0.34 ng/mL, respectively. Comparing to the control cohorts, the concentrations of both polyamines in the experimental cohorts exhibited statistically significant increases ($p < 0.01$). The expression of *Pt* gene encoding polyamine transporter protein was sharply up-regulated in cucumber roots after treatment with DJ bacteria under salinity-alkalinity stress; the expression was more than tenfold higher than that in the control groups. The enzyme activities of POD, SOD, and CAT in cucumber seedlings were higher compared to those in the control groups ($p < 0.01$). The stem height, number of lateral roots, and fresh weight of cucumber seedlings in the DJ treatment groups were 6.0 cm, 17 roots, and 0.42 g, respectively, whereas those of the control groups were 3.8 cm, 14 roots, and 0.28 g, respectively, with a notable difference between two cohorts ($p < 0.01$).

Conclusions DJ bacteria can live in salinity-alkalinity conditions by upregulating the expression of genes associated with chemotaxis. The resistance of cucumber seedlings under salinity-alkalinity conditions through the antioxidant pathway was increased by polyamines produced by DJ bacteria.

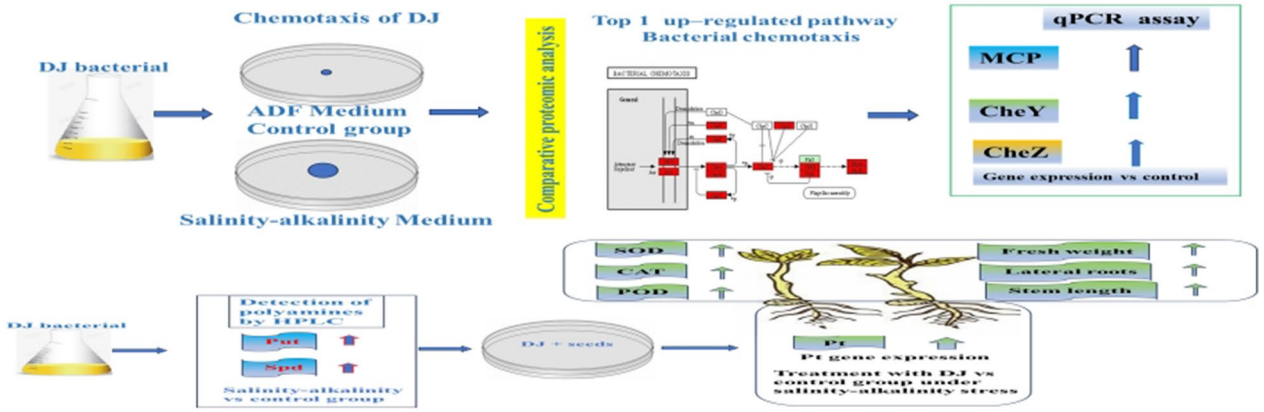
Keywords *E. cloacae* DJ, Proteomic analysis, Bacterial chemotaxis, Plant growth promotion

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Graphical Abstract



Introduction

Population growth and global environmental changes pose significant challenges to agriculture sustainability. Plant growth-promoting rhizobacteria (PGPR) are safer alternatives to chemical fertilizers and pesticides, because they reduce environmental pollution and result in high crop yields [1–4].

Plant growth-promoting bacterial species are common in nature, and numerous species have been isolated and evaluated for their plant growth promoting properties. They can facilitate plant growth and development in different ways, compring nitrogen fixation within the rhizosphere and phytohormones production such as auxins, cytokinins, and gibberellins, accelerating environment-originated nutrients uptake [5–9]. Polyamine production is another strategy that some bacteria use to promote plant growth. Studies have demonstrated that *Bacillus subtilis* OKB105 can produce and secrete spermidine, which induces growth and reduces ethylene levels in tobacco seedlings [10, 11].

Several researches have demonstrated that polyamine is an important compound found in fungi, bacteria [12, 13], mammals, and plants [14, 15]. Polyamines are involved in various processes such as gene expression [16], nucleic acid replication, protein synthesis, membrane stability, cell division, and differentiation [17, 18]. Recent studies have shown that exogenous polyamines have significant effects on plant abiotic stress. The exogenous application of polyamines helps to protect against damage induced by abiotic stresses. Exogenous spermidine (Spd) can induce Ca $(NO_3)_2$ stress tolerance in cucumber [14] and enhance photosynthetic and antioxidant capacities of citrus seedlings under high temperature [19]. Exogenous Spd also decreases the O_2^- generation rate and malondialdehyde content in tomato seedlings and alleviates salinity-alkalinity stress damage using antioxidant enzymes and non-enzymatic systems in chloroplasts [20]. Putrescine (Put) is an important small molecule and help to regulate plant growth in response to various environmental stresses. Exogenous application of Put has been reported to increase root length in strawberry microcuttings [21] and significantly increase abscisic acid content and chilling tolerance in tomato seedlings [22]. Combined Put treatment and arbuscular mycorrhizal fungus (AMF) inoculation significantly increased trifoliate orange seedling surface area, root length, root volume, and projected area than AMF treatment alone [23].

We isolated the polyamine-producing bacterial strain DJ from the rhizosphere soil of a plant (*Leymus chinensis (Trin.)* Tzvel) in the western region of Jilin Province, and 16S rDNA sequence analysis indicated that the bacterium was *Enterobacter cloacae*. Previous studies have shown that DJ bacteria could promote the growth of cucumber seedlings under salinity-alkalinity stress, and high-performance liquid chromatography analysis showed that putrescine and spermidine content in the supernatant of the bacterium was elevated under salinity-alkalinity conditions. Hence, we hypothesized that DJ bacteria can adapt to salinity-alkaline conditions and that the elevated levels of putrescine and spermidine in the bacterial supernatant under salinity-alkaline conditions may be related to their growth-promoting effects. The objective of this study was to elucidate the adaptive mechanism of DJ bacteria under salinity-alkalinity conditions and the underlying mechanism by which DJ bacteria improve the resistance of cucumber seedlings to salinity-alkalinity stress.

Experimental

Chemical and reagents

Acquisition of various research materials was as follows: Thermo Fisher Scientific (Waltham, MA, USA) provided putrescine, spermidine, acetonitrile, the BCA assay kit, and SYBR Green PCR Real Master Mix. Tiangen Biotech Co. Ltd. (Beijing, China) provided the RNA extraction kit, the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) supplied the superoxide dismutase (SOD) Assay Kit, catalase (CAT) Assay Kit, and peroxidase (POD) Assay Kit. All other chemicals, reagents were from Sangon Biotech Co., Ltd. (Shanghai, China).

Bacterial strains

Plant growth-promoting bacteria strain DJ (*E. cloacae*), isolated from the rhizosphere of *L. chinensis*, were stored at – 70 ℃. The ADF medium for DJ cultivation were DF (Dworkin and Faster) [24] medium contain 2 g arginine as the solo nitrogen source. Salinity-alkaline Medium: ADF medium with 1.755 g NaCl, (pH9).

The bacteria were kept at 28 ℃ on rotary shaker (150 rpm), and the biomass was monitored with a microplate reader (Flex Station 3, Molecular Devices) using a spectrophotometer 600 nm.

Growth of DJ bacteria in media of different pH conditions

The DJ bacteria were cultured with ADF medium at different pH values of 4, 5, 7, 8, and 9 at 28℃, respectively on a rotary shaker (150 rpm). The biomass of the DJ bacteria was measured at 2 h, 4 h, 8 h, 12 h, and 24 h of incubation, and the pH changes in the DJ bacteria during incubation were monitored at 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h via microplate reader (OD_{600}).

Swimming motility of DJ bacteria in salinity-alkalinity conditions

An agar powder of 0.28% was added to media and sterilized at 121 ℃ for 30 min. While the semi-solid medium was being cooled, 0.5 μL of DJ solution was inoculated in the center of the culture and then incubated at 28 ℃; the diameter of the colonies was measured after 24 h of incubation.

Comparative proteomic analysis

Protein sample preparation

The microorganisms were cultured using ADF and saline-alkaline growth media at 28 ℃, agitating at 150 rpm for 12 h separately. Following this, the microbial samples got snap-frozen within liquid nitrogen, pulverized, and subsequently transferred into receptacles. The samples were subjected to lysis, utilizing 300 μL of lysis solution with the addition of 1 mM phenylmethanesulfonyl fluoride. To facilitate lysis, the samples were treated with sonication, after which they were sent for centrifugation at 15,000 g for 15 min to eliminate any insoluble components. We also measured protein concentration by bicinchoninic acid (BCA) assay, samples were then portioned and preserved at – 80 ℃.

SDS-PAGE electrophoresis

10 μg proteins from each sample got obtained and sent for electrophoresis using a 12% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). The gel for separation was treated with crystal violet staining, incubated for 2 h, and then submerged for 12 h. Subsequent to staining, the gels were rinsed with distilled water until the bands became discernible. The stained gels were captured with an automated digital gel imaging system (Tanon 1600).

Digestion

Based on the protein concentration, 50 μg of proteins were obtained from each sample, and various sets of specimens were homogenized with lysis buffer to achieve uniform concentration. An appropriate volume of Dithiothreitol (DTT) got introduced into solution, resulting in final DTT 5 mM concentration, followed by a 30 min incubation at 55 ℃. Iodoacetamide of corresponding volume got suppled into the final 10 mM concentration, this mixture got kept within darkness for 15 min at room temperature. Subsequently, it was subjected to a sixfold dilution with pre-chilled acetone to promote protein precipitation. Samples got centrifuged at 8000 g for 10 min at 4 ℃ and reconstituted with triethylammonium bicarbonate (TEAB) 100 μL at a concentration of 200 mM. Trypsin was introduced according to a 1:50 mass ratio (trypsin: protein), and the solutions underwent a 12 h digestion at 37 ℃.

TMT labelling

The desiccated specimens were reconstituted in 100 mM TEBA 50 μL, and 88 μL of acetonitrile were introduced into a tandem mass tag (TMT) reagent vessel under room temperature conditions. We put centrifuged reagents to dissolve for 5 min, then mixed before a centrifugation step, which was repeated once more. Subsequently, TMT tag reagent 41 μL got combined with each sample for thorough blending. We incubated tubes at room temperature for 1 h. In order to halt reaction, 5% hydroxylamine 8 μL got suppled to each sample, and left to incubate for 15 min. The labeled peptide solutions got dehydrated and preserved at – 80 ℃.

HPLC analysis

Chromatographic separation was carried out via high-performance liquid chromatography (HPLC) utilizing an Agilent 1100 HPLC System equipped with an Agilent

Zorbax Extend RP column (5 μm, 150 mm × 2.1 mm). Two mobile phases, A (comprising 2% acetonitrile in HPLC-grade water) and B (consisting of 90% acetonitrile in HPLC-grade water), got employed for RP gradient. The tryptic peptides got isolated for 300 μL/min, their detection was performed at 210 and 280 nm. Eluted peptides were subjected to lyophilization to prepare them for subsequent mass spectrometric analysis.

Mass spectrometry analysis

Mass spectrometric analyses got conducted utilizing a Triple TOF 5600 mass spectrometer (SCIEX, USA), which featured a Nanospray III source (SCIEX, USA). For sample introduction, a capillary C18 trap column (3 cm × 100 μm) was employed, followed by separation on a C18 column (15 cm × 75 μm) utilizing an Eksigent nanoLC-1D plus system (SCIEX, USA). 300 nL/min flow rate was maintained. Buffer A contained 2% acetonitrile and 0.1% formic acid, meanwhile for buffer B it's 95% acetonitrile, 0.1% formic acid. Mass spectrometry scans got performed over a range from 375 to 1500, 250 ms accumulation time. The acquisition included MS peaks with intensities exceeding 260 and charge states spanning from 2 to 5. Collision-induced dissociation fragmentation for MS/MS spectral acquisition employed a rolling collision energy voltage. Masses were dynamically excluded for a 30 s interval to prevent reanalysis.

Database search

For comprehensive data analysis, the ProteinPilot software (v.5.0) was employed to conduct an extensive search of all unprocessed MS/MS data acquired from the TripleTOF 5600 instrument against reference protein database. The search criteria included trypsin digestion specificity and cysteine alkylation as a database parameter. To ensure data quality, a global false discovery rate below 1% was set, and quantifying peptide cohorts needed to comprise a minimum of two peptides.

Chemotactic gene expression analyses

The extraction of total RNA was executed utilizing the RNAprep Pure Bacteria Total RNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). To proceed, RNA samples underwent reverse transcription into complementary DNAs (cDNAs) as per the manufacturer's protocols (Tiangen Biotech Co., Ltd., Beijing, China). The synthesized cDNAs were harnessed as templates for polymerase chain reaction (PCR) amplification. Real-time quantitative PCR (qPCR) was carried out employing the ABI 7500 instrument (Applied Biosystems, USA) and the SYBR Green PCR Real Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA), following the manufacturer's instructions. 16S rDNA served as internal control, relative bacteria DJ gene expression was analyzed by $2^{-\Delta\Delta CT}$ method. The primer sequences employed for PCR got showed as Table 1.

Detection of polyamines using HPLC

The supernatant of the bacterium was harvested, cultivated with ADF and Salinity-alkaline Medium as described in the experimental methods section, and then dried under a freeze-vacuum dryer. The resulting residue underwent dissolution in 5 mL of ultrapure water, and resultant sample 2 mL got added to 10 mL centrifuge tube. To this, 1 mL of NaOH (2 mol/L) and 20 μL of benzoyl chloride were added, followed by vortexing and thorough mixing for 30 s. Subsequently, the mixture was incubated in a water bath at 37 ℃ for 20 min, while intermittent shaking at 5 min intervals.

After the derivatization step, 1 g of NaCl and 2 mL of ether were subjected to vortexing and mixing for 30 s. The upper ether layer was carefully transferred to a 5 mL centrifuge tube and then dried under nitrogen stream. Resulting sample was reconstituted in methanol 1 mL and filtered by nylon membrane (0.22 μm) using a vacuum setup subsequently. The prepared samples were subjected to analysis employing a Delta Pak C18 column (300 Å, 5 μm, 3.9 × 150 mm, Waters) joined with a high-performance liquid chromatograph (HPLC). In brief, within each sample 10 μL were extracted and injected into the column at 21.1 kg/cm^2 (300 psi), with mobile phases A (acetonitrile) and B (0.02 mol/L ammonium acetate) employed in the analysis.

Plant material and growth conditions

The bacterium DJ were cultivated with salinity-alkaline medium at 28 ℃ on a rotary shaker (150 rpm) for 12 h, and 20 cucumber (*Cucumis sativus* L.) seeds were soaked

Table 1 Primer sequences for quantitative PCR assay

Name	Primer sequence(5′—3′)
16S rDNA-F	GCACAATATTGCCCCCATCG
16S rDNA-R	GGCGTTGTAGTCACTGCTCT
MCP-F	CCATTGATGTTCCCACCCGT
MCP-R	CCTCACCTGTTCGCCAATCT
CheY-F	AGCAGAAGACGGCGTGGAT
CheY-R	GCAGCGGCAATGATGTTCTC
CheZ-F	AAACGCTGGGATGAGTGGTT
CheZ-R	GCAACTGAGCGTTGGTGAAG
GAPDH-F	TGCCGGAGATGAAGCCATTT
GAPDH-R	TAGCTCCAGCCTCAATGTGC
Pt-F	AAACCGTCCATCGTTTCCG
Pt-R	TTCCTCCTTGGCACCGTTAC

with the bacterial suspensions for 12 h. Uninoculated ADF medium was used as the negative control. Cucumber seeds were sterilized with 5% Nalco for 7–10 min, placed in 1/2 MS (Murashige-Skoog) medium after washing three times with distilled water, and 50 µL of filtered Na_2CO_3/$NaHCO_3$ (1:1, 10 mM each) mixed salt solution was added around the seeds. The plant seedlings were cultivated in a controlled plant growth chamber with a temperature set at 25 ℃, and they were subjected to a photoperiod of 16 h of cool fluorescent light followed by 8 h of darkness. After 7–10 days, the fresh weight, lateral roots, and stem height of the cucumber seedlings were measured, and each treatment was replicated three times.

Pt gene expression and antioxidant enzymes analysis

Total RNA was isolated from cucumber roots employing an RNA Isolation Kit (Tiangen Biotech Co. Ltd., Beijing, China). Subsequently, the RNA extracts were subjected to reverse transcription to synthesize cDNA following the manufacturer's recommended protocols (Tiangen Biotech Co. Ltd., Beijing, China), Real-time quantitative PCR assays were conducted as previously described. To normalize gene expression, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was utilized as a control. The relative expression of polyamine transporter gene (ID: 101217286) in cucumber roots was figured out by $2^{-\Delta\Delta CT}$ method. Primer sequences are available in Table 1.

To analyze cucumber seedlings (7–10 dpi), samples got cryogenically frozen within liquid nitrogen, subsequently pulverized into fine powder. The proteins from the cucumber seedlings were extracted utilizing the buffer with 20 mM potassium phosphate (pH7.4) equipped. Antioxidant enzymes activities, inclusive of superoxide dismutase (SOD), peroxidase (POD), as well as catalase (CAT) were quantified using commercial assay kits referred from professional guidelines.

Data analysis

All conducted tests on DJ bacteria and cucumber seedlings were performed in triplicate. To test of the growth-promoting effect of DJ bacteria on cucumber, 20 cucumber seeds were treated in each replication. The results are expressed as means ± standard deviation (SD), and the means were considered significant when the *p*-values were < 0.05.

Results and discussion

Adaptation of DJ bacteria to alkaline conditions

The bacterial strain DJ was isolated from the rhizosphere soil of a plant (*Leymus chinensis (Trin.)* Tzvel) in the western region of Jilin Province, where the soils are saline and alkaline, such that we hypothesized that the DJ strain must be tolerant to alkaline environments. To test our hypothesis, the biomass of DJ bacteria under different pH conditions was examined at different incubation periods. Results (Fig. 1A) showed that the biomass growth trend of DJ bacteria was basically the same under neutral and alkaline culture conditions and was significantly higher than that under acidic culture conditions. Several studies have demonstrated that the range of pH changes affects the activity of bacteria, so that the absorption of elements is affected by the pH of the growth environment [25, 26]. Usually, in acidic pH, the solubility of toxic elements increases and has a negative effect on the growth of bacteria [27].

The changes in pH of the bacterial cultures of pH7 and pH9 were also monitored during the incubation process. Figure 1 B indicated that the potential of hydrogen in the DJ bacteria culture tended to decrease from 2 to 24 h and

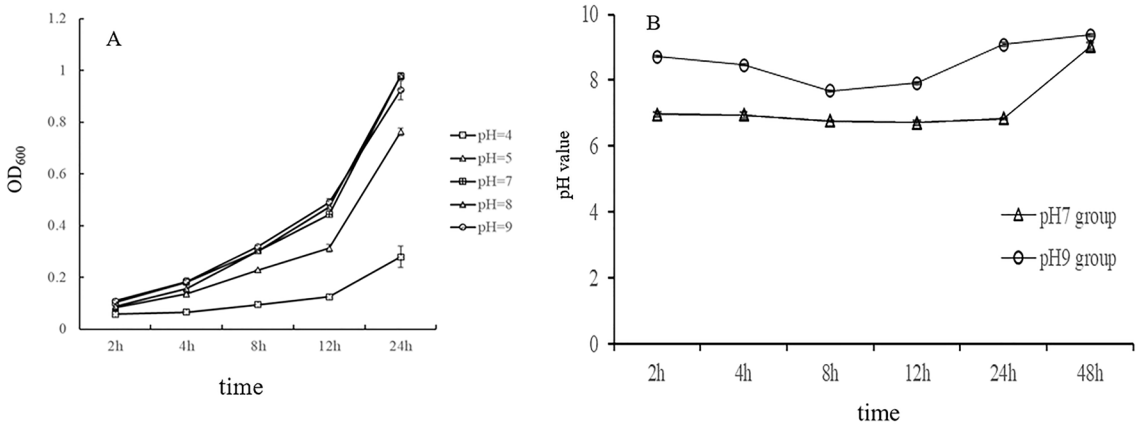


Fig. 1 Growth of bacterial strain DJ in media with different pH **A**. Bacterial biomass in media with different pH. **B**. Changes in the pH of bacterial cultures of pH7 and pH9 during the incubation process

gradually increase from 24 to 48 h, approaching pH9. These findings suggest that DJ bacteria can suitably live in alkaline environments.

Swimming motility of DJ bacteria under salinity-alkalinity conditions

As bacterium DJ grows well under alkaline conditions, we speculated that it may show some form of salinity-alkalinity. To address this issue, we used the swimming plate assay, which can measure the chemotactic response of bacteria to analyze the chemotaxis of DJ bacteria under salinity–alkalinity conditions [28]. DJ bacteria were inoculated in ADF and salinity–alkalinity medium, respectively. The average diameter of the colonies on the salinity–alkalinity medium was 3.3 cm, and this was significantly different ($p < 0.01$) from that (1.9 cm) of the control group (Fig. 2). Different soil types can influence the effectiveness of PGPR, and bacteria can sense environmental changes and move toward niches that are optimal for their survival and growth [29, 30]. The swimming motility of DJ bacteria in salinity–alkalinity medium was greater than that in ADF medium, indicating that DJ bacteria adapted to grow in salinity–alkalinity conditions.

Comparative proteomic analysis

Proteomic analysis was performed to elucidate the mechanism involved in the chemotaxis shown by the DJ bacterial strain towards salinity–alkalinity conditions. Comparative proteomic analysis revealed a total of 188 differentially expressed (116 up-regulated and 72 down-regulated) proteins in salinity-alkalinity relative to control groups (Fig. 3A, B). To gain a deeper understanding of the molecular and operational characteristics of these biomolecules, we conducted GO (Gene Ontology) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis on cohorts representing both the control and salinity-alkalinity experimental sets. The ascertained proteins were subsequently categorized into distinct groups according to their functional annotations (Fig. 3C, D). The top 20 of the up–regulated pathways of KEGG enrichment included bacterial chemotaxis, two-component system, flagellar assembly, butanoate metabolism, and quorum sensing. This study focused on the signaling pathways associated with bacterial chemotaxis. The signaling pathway consists of the transmembrane chemotactic receptor protein MCP, which senses chemical concentrations in the environment, and six regulatory proteins in the cytoplasm, connected by the key node CheY protein. MCP senses chemical stimuli and amplifies signals. Regulatory proteins receive chemical signals and transmit them to flagellar motors via CheY. CheZ is a phosphodiesterase that accelerates the dephosphorylation of CheY-P, decreases its binding to FliM, and shifts the flagellum from clockwise to counter-clockwise, allowing the bacteria to swim toward a favorable environment [31–33]. Comparative proteomic analysis showed that the expression of bacterial chemotaxis-related proteins was significantly upregulated under salinity-alkalinity conditions.

Validation of bacterial chemotaxis-related gene expression at the transcriptional level

According to proteomics analysis, the expression of proteins in signaling pathways associated with DJ bacterial chemotaxis was up-regulated, and the genes encoding for MCP, CheY, and CheZ in this signaling pathway were

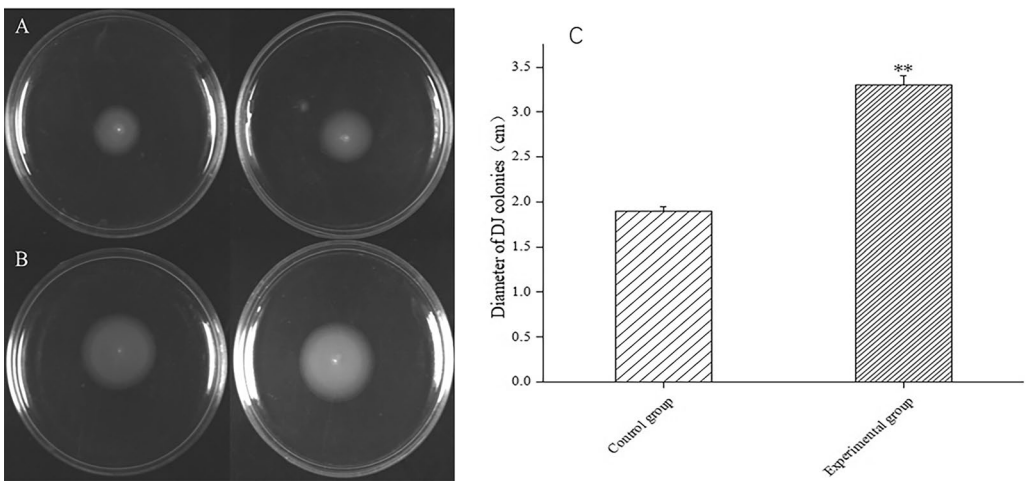


Fig. 2 Chemotactic responses of DJ to salinity-alkalinity conditions in swim plate assays. **A**. Colonies of DJ bacteria in ADF medium, **B**. Colonies of DJ bacteria in salinity-alkalinity conditions. **C**. Comparative results for the size of bacteria colonies in different conditions. Means ± SDs (n = 3). $^{**}p < 0.01$ vs control group

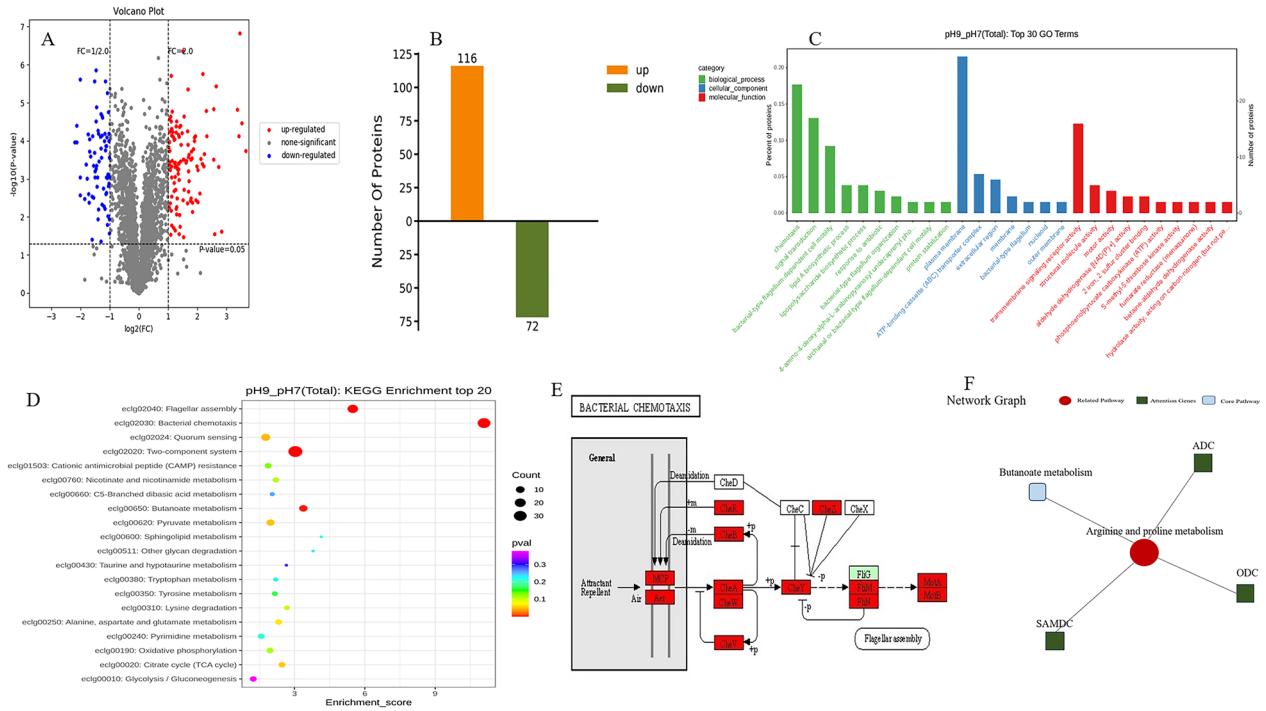


Fig. 3 Comparative proteomic analysis **A**. Volcano Plot of control groups versus salinity-alkalinity groups: red nodes indicate up-regulation; blue nodes indicate down-regulation. **B**. Up- and down–regulated proteins. **C**. GO enrichment in control groups versus group salinity-alkalinity groups. **D**. Top 20 up–regulated pathways of KEGG enrichment. **E**. Bacteria chemotaxis pathway. **F**. KEGG enrichment and related pathway analysis of ADC, ODC and SAMDC proteins. Differentially expressed proteins criteria: |log2(fold change) |> 1.5, $p < 0.05$

selected for further validation at the transcriptional level. The findings indicated an elevation in gene transcriptions under salinity-alkalinity conditions in comparison to the control group (Fig. 4), corroborating the outcomes of the proteomic analysis.

Swimming motility assay indicated that DJ bacteria adapted to grow in salinity-alkalinity conditions, and the results of proteomic analysis and validation experiments in the aspect of transcription indicated that genes expressions associated with bacterial chemotaxis was upregulated under salinity-alkalinity conditions. These findings imply that DJ bacteria increased swimming motility by regulating gene expressions related to chemotaxis and thus adapting to salinity-alkalinity environments.

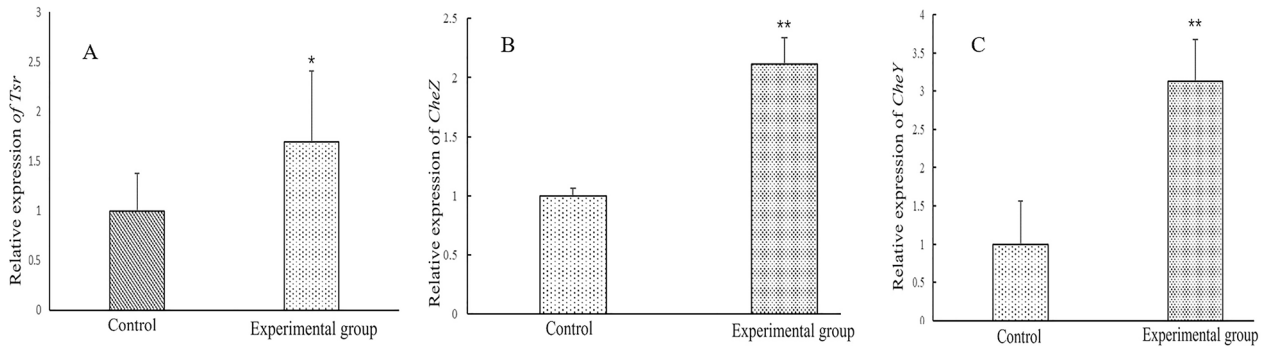


Fig. 4 Relative transcriptions level of genes *Tsr* (encoding for MCP), *CheZ* and *CheY* **A**. Relative transcriptions of *Tsr*. **B**. Relative transcriptions of *CheZ*. **C**. Relative transcriptions of *CheY*. Control groups: DJ bacteria were cultivated with ADF medium, experimental groups. DJ bacteria were cultivated with salinity-alkalinity medium. Means ± SDs (n = 3). *$p < 0.05$, **$p < 0.01$ vs control group

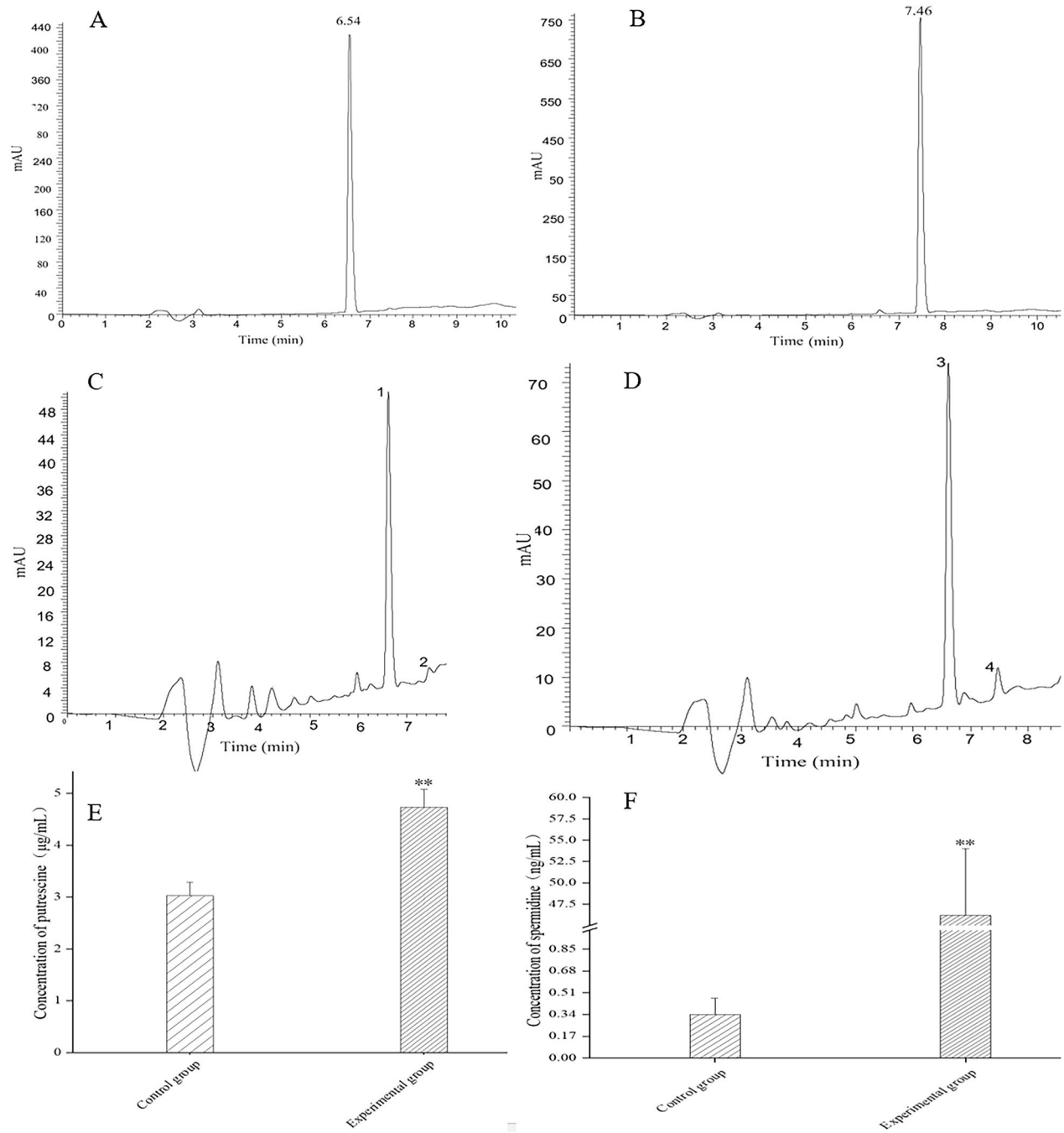


Fig. 5 Determination of putrescine and spermidine in supernatants of DJ using HPLC **A**. Putrescine standard. **B**. Spermidine standard. **C**. Putrescine and spermidine in supernatants of DJ cultivated with ADF medium; peaks: 1, putrescine, 2, spermidine. **D**. Putrescine and spermidine in supernatants of DJ cultivated with salinity-alkalinity medium; peaks: 3, putrescine, 4, spermidine. **E**. Comparative changes in putrescine contents under salinity-alkalinity conditions. **F**. Comparative changes in spermidine contents under salinity-alkalinity conditions. Means ± SDs (n = 3), $^{**}P < 0.01$ vs control group

Elevated levels of putrescine and spermidine in DJ bacterial supernatant under salinity-alkalinity stress

Arginine decarboxylase (ADC), Ornithine decarboxylase (ODC) and SAM decarboxylase (SAMDC) are key enzymes of polyamine biosynthesis in bacterial. KEGG enrichment and related pathway analysis of ADC, ODC and SAMDC proteins showed that these proteins all respond to the arginine and proline metabolism

Table 2 Growth promotion of cucumber seedlings by DJ bacteria under salinity-alkalinity stress

	Mean value	
	Control	**Experimental group**
Fresh weight (g)	0.28 ± 0.01825	0.42 ± 0.0241
Lateral root (n)	14 ± 3.84	17 ± 4.0258
Stem length (cm)	3.81 ± 0.7930	6 ± 0.6664

Means ± SDs (n = 20)

**$p < 0.01$ vs control group

pathway (Fig. 3F), which is the upstream pathway of the butanoate metabolism pathway. Because D5066_12500, DFS27_2442 and ssdA proteins in butanoate metabolism pathway were up-regulated, we speculated that the up-regulation of ODC, ADC, and SAMDC caused the up-regulation of the arginine and proline metabolism pathway, then affected butanoate metabolism, which resulted in a significant up-regulation of D5066_12500, DFS27_2442, and ssdA proteins in the butanoate metabolism pathway, ultimately resulting in changse in polyamine contents, which were obtained in the supernatant of the bacterium for detection by HPLC.

Analysis of polyamine content revealed significant differences under different conditions (Fig. 5, **$p < 0.01$). The content of putrescine and spermidine (4.73 μg/mL and 46.18 ng/mL) in the supernatant were significantly different in salinity-alkalinity medium; however, in the ADF medium, they were 3.02 μg/mL and 0.34 ng/mL, respectively. Put and Spd are the most common polyamine species found in bacteria [13]. Research has demonstrated that in some group of bacteria, Put is produced in response to acid stress [23] and can promote the growth of some bacteria [12]. DJ bacteria were cultured in different media, and the concentration of the bacteria was monitored via absorbance (OD_{600}) after 12 h: the result showed that the concentration was basically the same in ADF and salinity-alkaline media (Fig. 1), indicating that DJ bacteria responded to salinity-alkaline conditions and

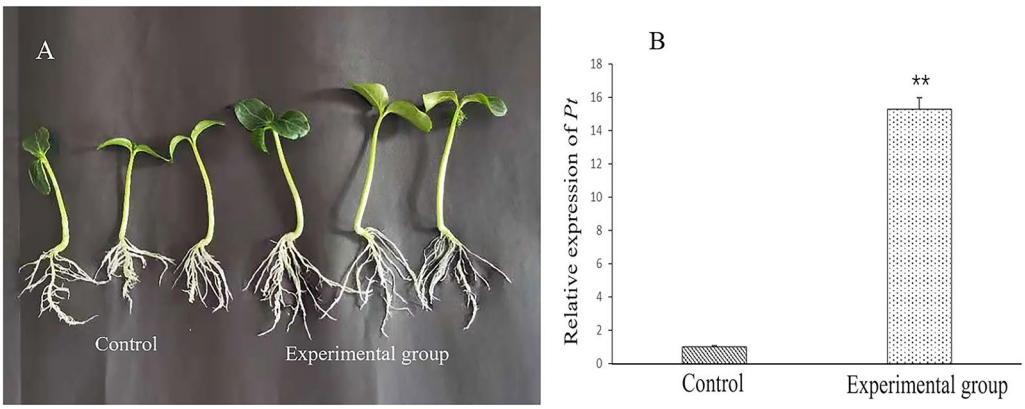


Fig. 6 Elevated expression of *Pt* gene in roots of cucumber seedlings after treatment with DJ bacteria **A**. Cucumis seedlings. **B**. *Pt* gene expression is elevated after treatment with DJ bacteria under salinity-alkalinity conditions. Means ± SDs (n = 3). **$p < 0.01$ vs control group

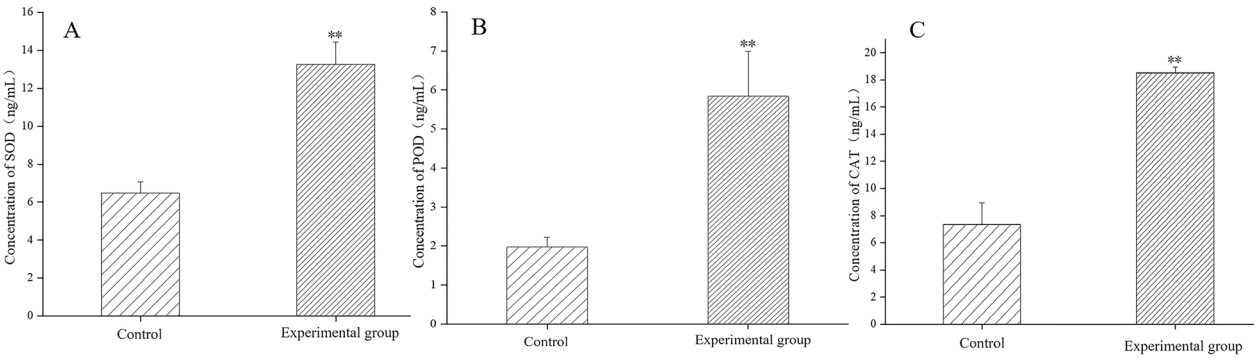


Fig. 7 Detection of antioxidant enzyme activities in cucumber seedlings under salinity-alkalinity conditions **A**. SOD. **B**. POD. **C**. CAT. Means ± SDs (n = 3). **$p < 0.01$ vs control group

resulted in elevated levels of putrescine and spermidine, which may have promoted the growth of DJ bacteria.

Mechanisms underlying the promotion effects of DJ bacteria on cucumber seedling in salinity-alkalinity conditions

Some researchers have confirmed that exogenous polyamines have positive effects on plant development [34, 35]. The application of Put and Spd at different concentrations increased the root fresh weight, dry weight of roses plants (*Rosa hybrida* L. 'Herbert Stevens') [36]. Put application also improved rooting frequency and promoted root elongation in *Arabidopsis thaliana* L. [37]. In addition, exogenous Put modulates shoot growth, and several studies have provided evidences that foliar application of Put significantly improved the plant height and number of leaves per plant [38, 39].

Several experiments in recent years have demonstrated that plant-promoting bacteria increase plant resistance to abiotic stress in various ways [40, 41]. This study assumed that DJ bacteria enhanced the resistance of cucumber seedlings to salinity-alkalinity conditions via polyamines in the supernatant. In this assay, the stem height, number of lateral roots, fresh weight of cucumber seedlings was examined, and the mean value of these indicators in DJ treatment groups were 6.0 cm, 17 roots, and 0.42 g, respectively, whereas in the control groups, they were 3.8 cm, 14 roots, and 0.28 21 g, respectively, indicating a notable difference between the DJ treatment and control cohorts (Table 2. $p < 0.01$).

To further determine how exogenous polyamines act in plants, the transcriptional level of the *Pt* gene (polyamine transporter protein, a protein that recognizes polyamines and specifically mediates the transport of polyamines across membranes) in seedling roots was examined using quantitative PCR. The control for standardization in this analysis was the *GAPDH* gene, and the findings unveiled a substantial upsurge in *Pt* gene expression within the cucumber seedlings' roots (Fig. 6B, $p < 0.01$). Polyamine substances in the bacterial supernatant interacted with polyamine transporter protein and facilitated the development of cucumber seedlings. To further explore relevant mechanisms, antioxidant enzymes were determined in cucumber seedlings.

Different abiotic stress factors increased ROS production and caused ROS-associated injury [42]. Polyamines have been established as advantageous agents in the amelioration of reactive oxygen species (ROS) and initiation of antioxidant machinery during challenging circumstances, consequently bestowing an extensive array of resilience against diverse stresses [14]. The plant's antioxidant system predominantly comprises SOD, POD, and CAT enzymes, with their functional levels serving as barometers of the plant's vulnerability to external adversities. These three enzymes work synergistically to maintain the free radical content in plants at a homeostatic level and prevent physiological and biochemical changes caused by free radicals [14, 20, 42]. This investigation entailed the evaluation of SOD, POD, and CAT activities within cucumber seedlings through specialized Assay Kits. Intriguingly, the outcomes unveiled significantly augmented antioxidant enzyme activities within the cucumber seedlings subjected to salinity-alkalinity stress and treated with DJ bacteria when juxtaposed with the control group (Fig. 7, $p < 0.01$).

Conclusions

We isolated the plant growth-promoting bacterium DJ (*E. cloacae*) from a salinity-alkalinity environment. The bacterial strain DJ showed a clear chemotactic response towards the salinity–alkalinity medium. This finding was further confirmed by comparative proteomic analysis, which revealed 188 differentially expressed proteins (116 upregulated and 72 downregulated) in the salinity-alkalinity groups compared to the control groups. Moreover, signaling pathway analysis showed that the expression of bacterial chemotaxis-related proteins was significantly upregulated under salinity-alkalinity conditions. Transcript analysis of DJ bacterial chemotaxis-related genes corroborated these findings. Increased levels of putrescine and spermidine in the supernatant of DJ bacteria were detected under salinity-alkalinity conditions. The expression of *Pt* gene encoding the polyamine transporter protein was sharply up-regulated in cucumber roots after treatment with DJ bacteria under salinity-alkalinity stress, and the enzyme activities of POD, SOD, and CAT were higher compared to those in the control groups. These results suggest that putrescine and spermidine in the supernatant of DJ bacteria acted as exogenous polyamines and interacted with polyamine receptor proteins in the cucumber roots, thereby leading to the increase in the activity of antioxidant enzymes, which in turn increased the resistance of the cucumber seedlings to salinity-alkalinity stress.

Abbreviations

PGPR	Plant growth-promoting rhizobacteria
ADF	DF (Dworkin and Faster) medium contain 2 g arginine
POD	Peroxidase
SOD	Superoxide dismutase
CAT	Catalase
SDS-PAGE	Sodium dodecyl sulfate—polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
DTT	Dithiothreitol
TEAB	Triethylammonium bicarbonate
TMT	Tandem Mass Tag
qPCR	Quantitative polymerase chain reaction
SYBR	Synergy brands
MS	Murashige-Skoog

SD	Standard deviation
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
MCP	Methyl-accepting chemotaxis protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Pt	Polyamine transporter protein
ROS	Reactive oxygen species
Put	Putrescine
Spd	Spermidine
HPLC	High-performance liquid chromatography
BCA	Bicinchoninic acid
ADC	Arginine decarboxylase
ODC	Ornithine decarboxylase
SAMDC	SAM decarboxylase

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Author contributions
XZN was mainly responsible for project management, YJF designed the research and wrote the manuscript, YXL was a major contributor to data analysis, HNW conducted the DJ related experiments, ZZ and ZYZ performed the assays of cucumber plants. All authors read and approved the final manuscript.

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