

# Salinity-responsive key endophytic bacteria in the propagules of *Kandelia obovata* enhance salt tolerance in rice

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**Abstract** Salinity is a major environmental stress affecting crop growth and productivity globally. The application of halo-tolerant plant growth-promoting bacteria (HT-PGPB) has been widely recognized to promote crop growth and reduce the adverse effects of salt stress. Herein, key endophytic bacteria that can respond to salinity changes were identified by analysis of the microbial community in propagules of *Kandelia obovata*. *Delftia tsuruhatensis* DYX29, a strain able to grow normally under high salinity conditions with a sodium chloride (NaCl) concentration of 5% (w/v), was obtained by pure culture. DYX29 has the ability to produce siderophores with a siderophore unit value of 87.6% and ACC (1-aminocyclopropane-1-carboxylate) deaminase with 29 U L<sup>-1</sup> and can synthesize intracellular amino acids and auxin induced by high salinity. Inoculation with DYX29 can remarkably promote the salt tolerance of rice. Under salt stress, the addition of DYX29 effectively promoted the growth of rice seedlings through a variety of approaches. It increased the biomass of rice seedlings by 32.9% (dry weight) and promoted the accumulation of soluble sugars by 23.1%. It also increased CAT and POD activities in rice leaves by 37.8 and 88.2%, respectively. Moreover, it maintained ionic homeostasis in roots and leaves. In addition, it upregulated the expression of growth-promoting hormones in roots, such as IAA, BL, ABA and SA, in rice roots by 27.8, 69.5, 123.7 and 28.6%, respectively. This study provides inspiration for screening valuable salt-tolerant pro-biotic bacteria from mangrove ecosystems for crop growth promotion under salt stress. It may also provide useful references for the development of new salt-tolerant and pro-biotic biofertilizers and the investigation of the related mechanism.

**Keywords:** salt stress, Halo-tolerant plant growth-promoting bacteria, *Delftia tsuruhatensis*,

microbial community<sup>1</sup>

## 1. Introduction

It has been widely recognized that the use of halo-tolerant plant growth-promoting microorganisms can promote crop growth and reduce the adverse effects of salt stress. Researchers previously found that inoculating isolated plant-associated microorganisms into nonhost plant species, including crops, can effectively enhance the productivity of the plants in adversity. As an example, Schmitz *et al.* (2022) isolated the core rhizosphere bacterial microbiome of the desert plant *Indigofera argentea* and constructed a representative SynCom (Synthetic community), which could be successfully inoculated into the rhizomicrobiome of tomato and promoted tomato growth under salt stress even in a nonsterile natural environment (Schmitz *et al.* 2022). Halo-tolerant microorganisms function in different ways for plant growth promotion. Abdelaziz *et al.* (2019) reported that the addition of the root endophytic fungus *Piriformospora indica* to the soil promoted an increase in tomato biomass under salt stress and lower Na<sup>+</sup>/K<sup>+</sup> values in shoots and roots through a mechanism associated with an increase in the level of *LeNHX1* in the leaves of tomato. Gond *et al.* (2015) disclosed that inoculation with *Pantoea agglomerans* significantly increased the total dry biomass of maize under salt stress through a mechanism of upregulated expression of the water channel protein-encoding gene family, especially the plasma membrane integrative protein (*ZmPIP*) gene. However, the exploration and utilization of halo-tolerant plant growth-promoting microorganisms are still far from sufficient. It is of high importance and great value to further expand the resources of halo-tolerant plant growth-promoting microorganisms for plant growth promotion under salt stress.

Mangrove ecosystems are located in the coastal habitats of tropical and subtropical intertidal estuaries and play an important role in maintaining the stability of bay and estuarine ecosystems, preserving biodiversity, preventing winds and fixing dikes, purifying water, and regulating the climate of cities (Feller *et al.* 2010; Lee *et al.* 2014). Mangrove forests have special ecological conditions, such as high salinity and low oxygen content, due to periodic seawater inundation. As

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a result of long-term natural selection and evolution, there are abundant salt-tolerant microbial resources in mangrove ecosystems (Mukherjee *et al.* 2019; Liu *et al.* 2020). *Kandelia obovata*, a member of mangrove forests, is a presuccessional intermediate species (Sheue *et al.* 2003). Viviparity in *K. obovata* is very common and is characterized by the direct germination of seeds on the parent plants without dormancy. Embryonic growth breaks through the seed coat and pericarp successively to form a propagule (Gentry 1987). Viviparity in mangrove plants was first observed and recorded over a hundred years ago (Cook 1907) and has been believed to facilitate their adaptation to high salinity in coastal wetlands and to inhibit the outward dispersal of propagules, preventing their rapid occupation of intertidal ecological niches (Joshi 1933; McKee 1995; Han *et al.* 2011). The direct and prolonged contact between the propagules of *K. obovata* and their parent make it possible for the phyllospheric microorganisms in the parent, which have been adapted to the mangrove environment, to colonize the propagules (Kim *et al.* 2020). It has been indicated that plant hosts are highly dependent on vertical transmission symbionts for nutrient uptake and utilization. The environmental adaptation of hosts is significantly reduced when vertical transmission symbionts are eliminated (Fisher *et al.* 2017). However, there are few reports on the composition and function of the microbial community in the propagules of *K. obovata*, and it remains unknown how the endophytic microbial taxa of vertical transmission promote the adaptation of propagules to high-salinity environments after they fall into seawater. Therefore, the first objective of this work was to identify the key endophytic bacteria responsive to salinity in the propagules of *K. obovata*, which may provide potent halo-tolerant growth-promoting effects for crops under salt stress.

Currently, more than one-third of irrigated land in the world is affected by salinization (Zhang *et al.* 2019), which causes poor harvests of crops and threatens global agriculture. In particular, the vast area of saline-alkali land in China seriously affects the cultivation of rice, which is one of the most important crops in the country. To overcome this issue, one solution is to develop new varieties of saline-tolerant rice through breeding technologies. Another potent solution is to apply halo-tolerant growth-promoting microorganisms to help rice grow under salt stress. It was previously reported that inoculation with *Streptomyces albidoflavus* OsiLf-2, a moderately salt-tolerant endophytic actinomycete, increased the osmotic-adjustment ability of the rice host by increasing the proline content by 49.4% and soluble sugar content by

49.4% in rice under salt conditions (Niu *et al.* 2022). Sun *et al.* (2020) also reported that the increase in the fresh weight of rice Nipponbare (salt-sensitive) seedlings by *Pantoea alhagi* NX-11 was 30.3% under saline conditions. The second objective of this work was to apply halo-tolerant growth-promoting microorganisms obtained from *Kandelia obovata* to rice growth under salt stress and to explore the underlying mechanism.

In this work, the role of salt stress in regulating the microbial community of *K. obovata* propagules was first explored. Then, *Delftia tsuruhatensis*, a key species responding to salinity changes, was identified in terms of physiological and metabolic properties, and its halo-tolerant and growth-promoting effects on rice seedlings were analyzed. Furthermore, the underlying mechanism by which this bacterium promotes the salt tolerance and growth of rice was elucidated. This study provides an idea for screening new HT-PGPB resources from natural habitats and provides a useful reference for elucidating the halo-tolerant and growth-promoting mechanisms of other crops.

## 2. Materials and methods

### 2.1. Sites, sampling and environmental properties

In this study, sampling was conducted in the Shenzhen Bay Mangrove Wetland (22°30'-22°39'N, 113°53'-114°05'E), Guangdong, China. Sufficient *Kandelia obovata* propagules were randomly collected using sterile tools from different individual plants in April 2021. The Shenzhen Bay Mangrove Wetland covers an area of approximately 10,000 square hectometer. It has a subtropical marine climate with an average annual temperature of 22.4°C and an average annual rainfall of 1700-1900 mm (Yao *et al.* 2019).

### 2.2. Propagule culture

The collected propagules of *K. obovata* were placed in foam boxes equipped with seedling trays and hydroponically watered with Hoagland nutrient solution. The nutrient solution was changed weekly to ensure adequate nutrients in the water. After 30 days of incubation, most of the propagules germinated, roots developed, and the first pair of leaves was drawn out. Subsequently,

propagules with similar growth conditions were randomly divided into two groups and cultured under two different conditions: Hoagland nutrient solution without NaCl (Control) and Hoagland nutrient solution with 3% salinity (Treatment). The culture medium was changed regularly to keep the salinity and nutrients stable. After 60 days of culture, propagules were collected and stored at -80°C until use.

### 2.3. Collection of endophytic microbes from hypocotyls

The hypocotyl samples were treated as described by Nelkner *et al.* (2019) with some modifications. Briefly, hypocotyl teguments were surface-disinfected successively with sterile distilled water for 30 s, 70% ethanol for 2 min, 2.5% sodium hypochlorite (containing 0.1% Tween 80) for 5 min and 70% ethanol for 30 s, followed by 5 rinses with sterile distilled water for 2 min. The effectiveness of the disinfection procedure was evaluated by plating the last washing water on tryptic soy agar (TSA) plates. No colonies were obtained from any of the TSA plates after 6 days of incubation at 30°C. After disinfection, propagules were cut into small pieces with sterile scissors and crushed with a sterile mortar and pestle in liquid nitrogen. Part of the pulverized samples were used for subsequent endophytic bacterial screening, and the rest of the samples were stored at -80°C for subsequent DNA extraction.

### 2.4. Microbial community analysis

**DNA extraction and PCR amplification** Total DNA was extracted from pulverized hypocotyl samples using the FastDNA® Spin Kit for Soil (Mpbio, California, U.S. and then checked for purity and concentration. Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 region of the bacterial 16S rRNA gene. The amplification procedure was set as described by Frank *et al.* (2008), and then PCR products were detected and purified.

**Illumina MiSeq sequencing** Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

**Bioinformatics analysis** The raw 16S rRNA sequencing data were quality filtered, merged and clustered by OTU using Yang's method (Yang *et al.* 2023). Species classification information corresponding to each OTU was obtained based on the 16S rRNA database Silva V138.

**Diversity analysis** Alpha-analysis was carried out based on Chao1 and Shannon diversity measures at the OTU level. Beta-analysis was carried out using Bray–Curtis dissimilarity at the OTU level.

**Taxonomic composition** Taxa composition was generated at the phylum and genus levels. For phylum-level taxa, small taxa were merged into “others” with OTU counts <10. For genus-level taxa, the distribution of bacteria with the top 20 items was determined, and the rest of the genera were merged into “others.”

## 2.5. Identification of key endophytic bacteria

**Screening of key endophytic bacteria** The hypocotyl samples in Section 2.4 were ground and added to sterile water to prepare the endocotyl bacterial suspension. After proper dilution, the suspensions were coated on nutrient agar (NA) medium containing 5% sodium chloride and cultured at 30°C for 72 h. A single colony was selected and purified several times until a monoclonal colony was obtained.

**Molecular Identification** Bacterial DNA was extracted using a TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The 16S rRNA gene sequence was amplified using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') and sequenced by the Sanger method (Frank *et al.* 2008). The obtained 16S rDNA sequence was examined by the NCBI database using BLAST algorithms. The phylogenetic tree was constructed using the neighbor-joining method with MEGA 7.0 software.

### **Evaluation of salt tolerance and determination of the salt reduction efficiency of strains**

TSA media with NaCl concentrations (w/v) of 5, 10 and 15% were prepared, and a suspension of the tested strain with a cell density of  $OD_{600}=0.1$  was inoculated and cultured on a shaker for 72 h (30°C, 180 r min<sup>-1</sup>). Then, the  $OD_{600}$  of the fermentation broth was determined. At the same time, the salinity value of the culture medium with 5% NaCl concentration was measured with a salinometer. The relative siderophore content (SU value) was calculated according to Patel's

method (Patel *et al.* 2018).

**Assessment of PGP (plant growth promoting) attributes** Phosphate solubilization was qualitatively determined using Bashan's methodology (Bashan *et al.* 2013). The DYX29 strain was grown in phosphorus-free SRSM liquid medium supplemented with calcium phosphate. The soluble phosphorus converted by the strain was measured by UV–Vis spectrometry to assess its phosphate solubilization ability. Potassium dissolution was qualitatively determined using Wang's methodology (Wang *et al.* 2018). Atmospheric nitrogen fixation was qualitatively determined using Santos's methodology (Santos *et al.* 2001). Siderophore production was qualitatively determined using Dukare's methodology (Dukare and Paul 2021). ACC deaminase activity was detected by ELISA using the corresponding kit (Meimian, Jiangsu, China). Each experiment was replicated three times.

**Differential metabolite analysis of strains responding to salt stress** The strains were inoculated into normal and 5% (w/v) NB and named Del-0 and Del-5, respectively. The precipitated cells were collected by centrifugation, washed three times with precooled PBS solution, resuspended in 1 mL PBS solution and accurately counted to  $10^7$  cells in each EP tube. Liquid chromatography–mass spectrometry (LC–MS) was used to detect and analyze the intracellular metabolites of the strains under the two conditions (Li *et al.* 2018). It was conducted on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm×2.1 mm i.d., 1.8  $\mu$ m; Waters, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in water:acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5, v/v) (solvent B). The flow rate was 0.40 mL min<sup>-1</sup>, and the column temperature was 40°C. The injection volume was 3  $\mu$ L. The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode and negative mode. The optimal conditions were set as follows: source temperature at 425°C; sheath gas flow rate at 50 arb; aux gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at -3500 V in negative mode and 3500 V in positive mode. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 70-1050 m/z. The obtained metabolites were compared with the KEGG database. The metabolism data analysis was performed in the Majorbio online analysis platform (<https://cloud.majorbio.com>) (Majorbio, Shanghai, China).

## 2.6. Plant growth promotion assay on rice under salt stress

The surface of the rice seeds was disinfected as described previously by Yang *et al.* (2023). *D. tsuruhatensis* was cultured in nutrient broth (NB), and the precipitated cells were collected by centrifugation and washed twice with sterile water. The bacterial suspension was prepared with sterile water or 100 mmol L<sup>-1</sup> sterile NaCl solution, and the OD<sub>600</sub> was adjusted to 0.05. Rice seeds were soaked with 10 mL of the above bacterial suspension and soaked with the same amount of sterile water or 100 mmol L<sup>-1</sup> sterile NaCl solution as the control. Each treatment was repeated three times.

The root length, shoot length, seed germination index and seed vigor index of rice seedlings were measured on the 7th day of culture.

## 2.7. Determination of physiological and biochemical characteristics of rice under salt stress

Rice seeds were placed in a light incubator for hydroponic culture for two weeks and then divided into four groups (H<sub>2</sub>O, H<sub>2</sub>O+DYX29, 150 mmol L<sup>-1</sup> NaCl, and 150 mmol L<sup>-1</sup> NaCl+DYX29). After one week of treatment, samples were collected to determine the following indicators. The uppermost fully expanded leaves of rice were selected to determine the relative content of chlorophyll, and the SPAD value at the middle position of the leaves was measured by SPAD-502Plus (Konica Minolta, Japan). The soluble sugar content was measured by the anthrone colorimetry method following Niu's work (Niu *et al.* 2022). Malondialdehyde (MDA) content was determined by a previous method (Rao and Sresty 2000). The activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione reductase (GR) were measured with the corresponding assay kits (Cominbio, Suzhou, China) based on the manufacturer's protocols. The ion content was determined with an inductively coupled plasma atomic emission spectrometer (ICP–AES) following Niu's method (Niu *et al.* 2022). Phytohormone levels (IAA, BL, ABA, and SA) in rice roots were measured by liquid chromatography-tandem mass spectrometry (LC–MS/MS). The mobile phases consisted of 0.1% formic acid in water (solvent A)

and acetonitrile (solvent B). From 0-10 min, the ratio of mobile phases A to B was 95:5; from 10-15 min, the ratio of mobile phases A and B changed to 20:80; from 15-16 min, the ratio of mobile phases A and B changed to 0:100; from 16-18 min, the ratio of mobile phases A and B remained at 0:100; from 18-19 min, the ratio of mobile phases A and B changed from 0:100 to 95:5; and from 19-22 min, the ratio of mobile phases A and B remained at 95:5. The flow rate was 0.30 mL min<sup>-1</sup>, and the column temperature was 40°C. The injection volume was 3 µL.

## 2.8. Quantitative real-time polymerase chain reaction analysis

Total root RNA was isolated using the FastPure Universal Plant Total RNA Isolation Kit (Vazyme, Jiangsu, China). cDNA samples were prepared using a HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Jiangsu, China). The expression of key genes of salt tolerance in rice was analyzed by qRT-PCR, and *OsActin* was used as an internal control. The relative changes in gene expression were calculated by the  $2^{-\Delta\Delta CT}$  method. The RT-PCR assay consisted of three independent experiments with three replicates of each experiment.

The genes to be analyzed included osmoregulatory substance synthesis genes (*OsBADH1*), Na<sup>+</sup>/K<sup>+</sup> ion transporter genes (*OsNHX1*, *OSHKT1;1*) and plant growth hormone synthesis genes (*OsPIN1*, *OsYUCCA1*).

## 2.9. Statistical analysis

The data were analyzed using GraphPad Prism 8.0 with variance analysis. Normality and variance homogeneity were tested using the D'Agostino & Pearson test and Brown-Forsythe test, respectively. The data were normally distributed with homogeneous variance. A two-tailed Student's t test was used for significant analysis between the two groups. The mean values plus standard errors and significance levels were calculated (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ). Multigroup significance analysis was performed using Tukey's HSD test. Different letters indicate significant differences between different groups (\*,  $P \leq 0.05$ ).

## 2.10. Data availability

Raw sequence data for 16S rRNA reads were deposited into the NCBI Sequence Read Archive under Bioproject number PRJNA988464. The 16S rDNA sequences of strains *Delftia tsuruhatensis* DYX29 in this study were deposited in GenBank with accession numbers OQ976924.

### 3. Results

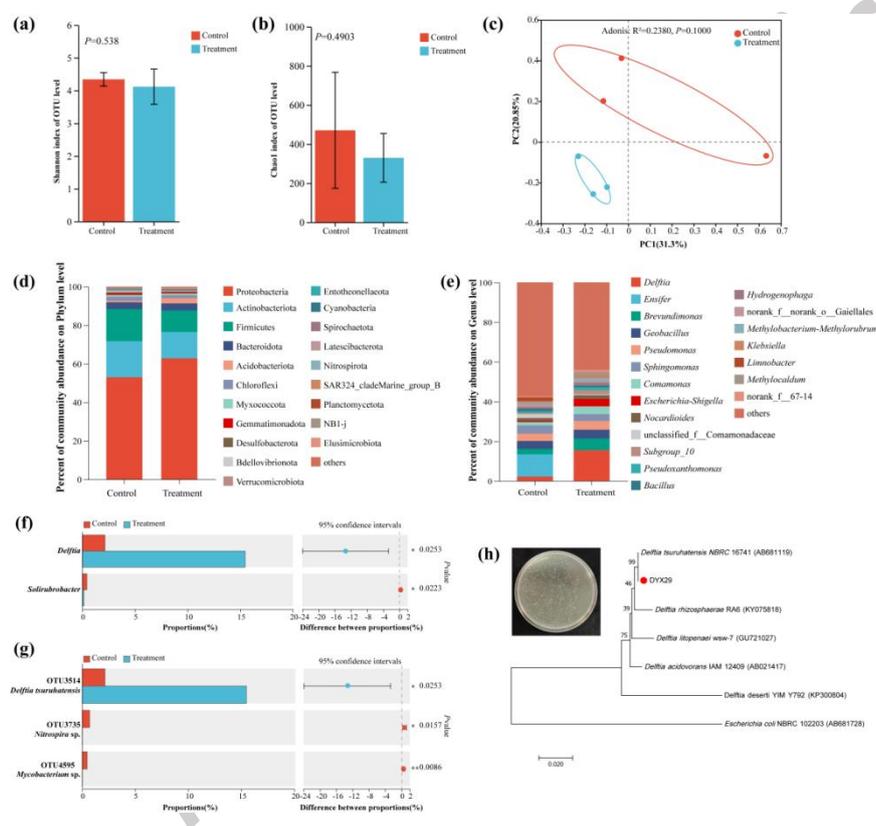
#### 3.1. Analysis of endophytic bacterial communities in propagules of *K. obovata* and identification of key endophytic bacteria in response to salinity

A total of 38 phyla, 107 orders, 236 orders, 391 families, 742 genera, 1127 species and 1512 OTUs were obtained by high-throughput sequencing analysis of the endophytic bacteria in the propagules of *K. obovata* under salt-treated and control conditions. As shown in Fig. 1-A and B, there was no significant difference between the diversity index (Shannon) and richness index (Chao1) of the endophytic bacterial communities in the propagules in the salt-treated group and the control group. PCoA analysis also showed that salt treatment had no significant effect on the structure of the endophytic bacterial community in viviparous seedlings ( $R^2=0.2380$ ;  $P$ -value=0.1000).

The structure of the endophytic bacterial community in the propagules of both groups of *K. obovata* was similar at the phylum level, and the number of dominant phyla with a relative abundance higher than 1% was six. The relative abundance from high to low was Proteobacteria, Actinobacteria, Firmicutes, Bacteroidota, Acidobacteriota and Chloroflexi (Fig. 1-D). At the genus level, the relative abundance in the two groups was consistent for the top 20 genera (Fig. 1-E). In addition, compared with the control group, the relative abundance of genera including *Ensifer*, *Sphingomonas*, *unclassified\_f\_\_Comamonadaceae*, *norank\_f\_\_norank\_o\_\_Gaiellales*, *Bacillus* and *Limnobacter* in the treatment group decreased, while the relative abundance of other dominant genera such as *Delftia*, *Brevundimonas* and *Geobacillus* increased. This indicated that salt treatment can affect the relative abundance of some dominant taxa.

Moreover, at the genus level, the relative abundance of *Delftia* was significantly higher in the treatment group (Fig. 1-F). At the OTU level, OTU3514 (*Delftia tsuruhatensis*) was also higher in the treatment group, while OTU3735 (*Nitrospira* sp.) and OTU4595 (*Mycobacterium* sp.) were significantly lower than those in the control group (Fig. 3-G). OTU3514 (*Delftia tsuruhatensis*) was

the only species belonging to *Delftia* in the overall OTU, and it showed significant differences at both the genus and OTU levels, with the largest variation between groups. Therefore, we proposed to obtain endophytes and target strains in the propagules of *K. obovata* by pure culture experiments. A total of 30 bacterial strains were obtained through morphological and molecular characterization, including the key species *D. tsuruhatensis* responding to salinity changes, named *D. tsuruhatensis* DYX29. The colony morphology of this strain is round, white and opaque in color, with rough surfaces and flat sections (Fig. 1-H). The optimal phylogenetic tree with a sum of branch lengths of 0.29 was obtained in MEGA7 using the neighbor-joining method (Fig. 1-H) and clustered in the same branch as *D. tsuruhatensis*.



**Fig. 1** Diversity of endophytic bacteria and identification of key species. A, Chao1 richness index. B, Shannon diversity index. C, principal coordinate analysis of community composition. D, relative abundance of phyla in different groups. E, significant difference test at the genus level. F, relative abundance of most prevalent genera in different groups. H, significant difference test at the OTU level. G, morphological characterization of strain DYX29 and phylogenetic tree. Error bars (mean $\pm$ SD,  $n=3$ ), tests for significant differences between groups for A, B, F and G were performed using two-tailed Student's *t*-test, and tests for C were performed using ADONIS. \*

indicates statistical significance (two-tailed Student's *t*-test, \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ).

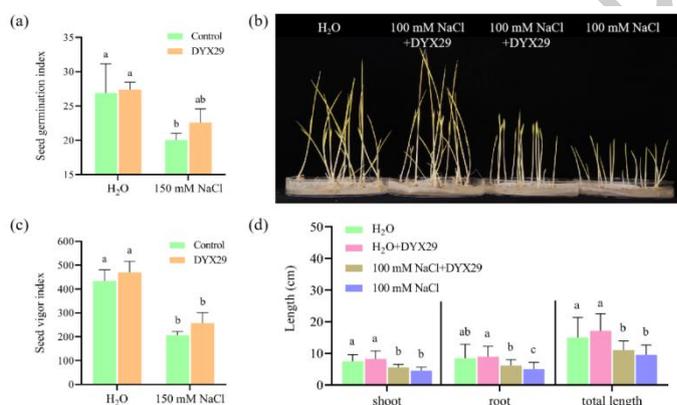
### 3.2. Physiological functions and metabolic characterization of *D. tsuruhatensis* DYX29

Whether *D. tsuruhatensis* DYX29 has the characteristics of salt tolerance, salt reduction and plant growth promotion is very important for its function. The results showed that *D. tsuruhatensis* DYX29 could grow normally in conditions with a salt concentration of 5% (w/v). Microorganisms consume a large amount of free  $\text{Na}^+$  in the environment during their growth and reproduction, which in turn reduces the concentration of salt ions in the environment and mitigates the toxic effects of high concentrations of salt ions on plants. We found that the salt ion content in the liquid medium could be reduced from 5 to 4.8% when strain DYX29 was cultured for 24 h. The PGP test revealed that the strain can produce siderophores with an SU value of 87.6%, belonging to the category of high siderophore-producing bacteria. Strain DYX29 also exhibited ACC deaminase activity.

In addition, non-targeted metabolomics analysis was performed on the intracellular products of *D. tsuruhatensis* DYX29 under normal culture conditions (Del-0) and culture conditions with a 5% salt concentration (Del-5). The results showed that a total of 1218 secondary metabolites were identified in cationic and anionic modes under Del-0 and Del-5 conditions. The identified metabolites were compared to the KEGG Compound database, and a total of 108 annotation results were obtained, among which the largest number of metabolite sets were annotated to amino acids (24 in total). After salt treatment, 143 metabolites of *D. tsuruhatensis* DYX29 showed significant changes. The metabolites whose abundance increased significantly after salt treatment are shown in Table S1, mainly including 5-nonyl-1,3-phenyldiol, glutamic acid, asparagine, lysine, uridine, 3-indoleacetic acid and other substances.

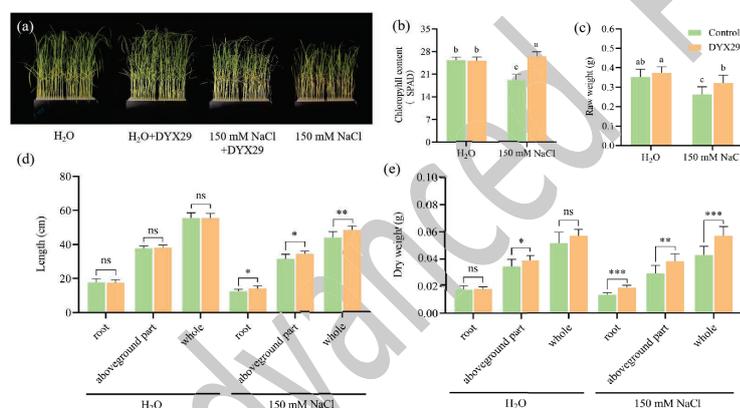
### 3.3 Effect of salt-tolerant endophytic bacteria on the growth of rice under salt stress

**Effects on rice seed germination** The results showed that both the seed germination index and vigor index were significantly lower under 100 mmol L<sup>-1</sup> NaCl treatment than those under H<sub>2</sub>O treatment (Fig. 2-A). In addition, the addition of strain DYX29 had no significant effect on either the seed germination index or vigor index under H<sub>2</sub>O treatment, whereas the addition of strain DYX29 was effective in increasing the seed germination index as well as enhancing the vigor index under 100 mM NaCl treatment (Fig. 2-A). After germination, the total length (sum of root and shoot lengths) of rice in the 100 mM NaCl group was significantly shorter than that in the H<sub>2</sub>O group (Fig. 2-B and C). Under H<sub>2</sub>O treatment, the addition of strain DYX29 did not significantly increase root length, shoot length or total length. In contrast, under 100 mmol L<sup>-1</sup> NaCl treatment, the addition of strain DYX29 also significantly increased root length but had no significant effect on shoot length and total length (Fig. 2-D,  $P < 0.05$ ). The above results indicated that the strain performed better in promoting the growth of rice under salt stress conditions.



**Fig. 2** Effect of *D. tsuruhatensis* DYX29 on the germination phenotype of rice seeds. A, seed germination index under different treatments. B, a representative photo of rice seed germination results. C, seed vigor index under different treatments. D, length of each part of rice seedling. Error bars (mean±SD,  $n=3$ ), different lowercase letters indicate significant differences (ANOVA, Tukey's HSD test).

**Effects on the growth of rice seedlings** Our results indicated that strain DYX29 was able to colonize the root hair region of rice roots but was unable to enter the rice plant. The effect of the strain on the growth of rice seedlings under salt stress conditions is shown in Fig. 3-A. Under H<sub>2</sub>O treatment, there was no significant difference between the length of various parts of rice with the added strain and that of the control group, while under 150 mmol L<sup>-1</sup> NaCl treatment, the root length, above-ground length and total length of rice were significantly increased after inoculation with the strain (Fig. 3-D). This result was consistent with the seed germination test. Additionally, there were no significant changes in fresh weight, dry weight of roots or total length of rice after inoculation with the strain under H<sub>2</sub>O conditions compared to the control group (Fig. 3-C and E). However, in the group under 150 mmol L<sup>-1</sup> NaCl treatment, the addition of the strain resulted in a significant increase in the overall fresh weight and dry weight of all parts of the rice (Fig. 3-C and E). Similarly, it also prompted a 37.8% increase in the SPAD values of rice leaves in salt treatment but had no significant effect on that under water conditions (Fig. 3-B).

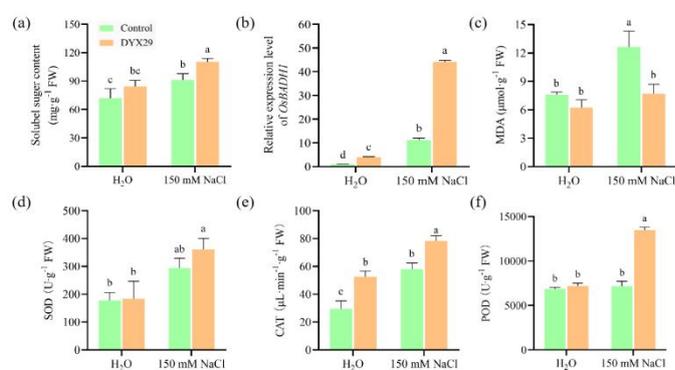


**Fig. 3** Effect of *D. tsuruhatensis* DYX29 on the rice seedling growth phenotype. A, photographs of the growth phenotype of rice seedlings. B, total fresh weight of rice seedlings. C, chlorophyll content of rice leaves. D, the length of each part of the rice seedling. E, dry weight of rice seedling parts. Error bars (mean±SD, n=12). \* indicates statistical significance (two-tailed Student's *t*-test, \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ). Different lowercase letters indicate significant differences (ANOVA, Tukey's HSD test).

### 3.4. Effects of *D. tsuruhatensis* DYX29 on physiological and biochemical indices of rice under salt stress

**Effects on osmoregulatory capacity, antioxidant system and ionic homeostasis in rice** Salt stress increases the osmotic pressure of plant cells by increasing the content of soluble sugars. The soluble sugar content of rice leaves under 150 mmol L<sup>-1</sup> NaCl treatment was significantly higher than that under H<sub>2</sub>O treatment (Fig. 4). The addition of the strain increased the soluble sugar content of rice in the H<sub>2</sub>O treatment by 17.3% and increased that in the group under 150 mmol L<sup>-1</sup> NaCl treatment by 23.1%. Meanwhile, the addition of DYX29 significantly increased the expression level of *OsBADH1*, which encodes a key enzyme of the betaine synthesis pathway, by 301.3 and 294.8% in both the H<sub>2</sub>O and 150 mmol L<sup>-1</sup> NaCl treatments, respectively. Betaine can act as an osmoregulator that schedules cellular responses to hyperosmolarity. These results suggested that DYX29 induced enhanced osmoregulation in rice.

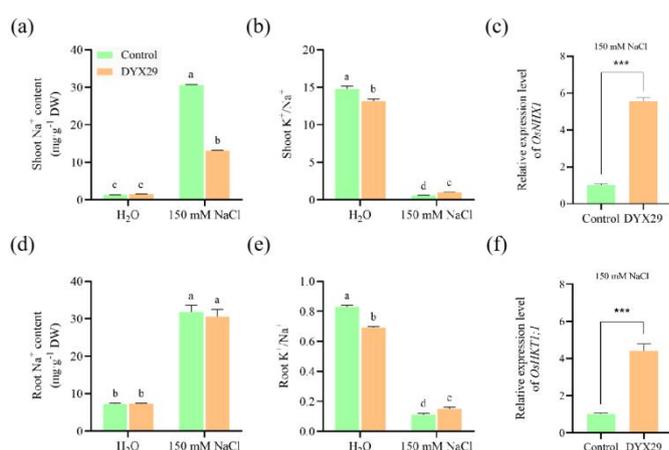
Plants undergo membrane lipid peroxidation under salt stress, as indicated by the presence of malondialdehyde. The addition of the strain significantly reduced the malondialdehyde content in rice roots under salt-treated conditions but had no significant effect under H<sub>2</sub>O conditions (Fig. 4-C). In general, plants under adverse conditions scavenge excess free radicals through the protective enzyme system, thereby reducing the extent of damage to the cell membrane structure. Under 150 mmol L<sup>-1</sup> NaCl treatment, the addition of DYX29 increased SOD, CAT and POD activities in rice leaves, where CAT and POD were significantly increased by 37.8 and 88.2%, respectively. This demonstrated that DYX29 improved the antioxidant capacity of rice.



**Fig. 4** Effect of *D. tsuruhatensis* DYX29 on osmotic regulation and antioxidant systems in rice. A, soluble sugar content. B, expression level of *OsBADH1*. C, MDA content. D, SOD activity. E, CAT activity. F, POD activity.

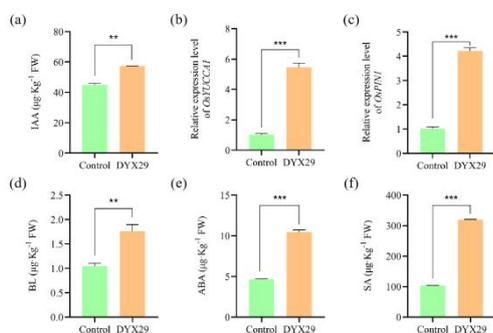
Error bars (mean±SD,  $n=3$ ). Different lowercase letters indicate significant differences (ANOVA, Tukey's HSD test).

In addition, the addition of DYX29 significantly reduced the  $\text{Na}^+$  concentration in rice leaves under 150 mmol  $\text{L}^{-1}$  NaCl treatment, whereas it had no significant effect on the  $\text{Na}^+$  concentration in roots (Figs. 5-A and D), but it promoted  $\text{K}^+$  accumulation in both groups, resulting in a significant increase in the  $\text{K}^+/\text{Na}^+$  ratio by 36.4% in leaves and 71.2% in roots (Fig. 5-B and E). *OsNHX1* and *OsHKT2;1* are genes involved in  $\text{Na}^+$  compartmentalization (Fukuda *et al.* 2011) and  $\text{K}^+$  transport (Wang *et al.* 2015), and the results showed that the addition of DYX29 significantly increased the expression levels of these two genes in rice roots under salt stress (Fig. 5-C and F), which together assisted in the maintenance of ionic homeostasis in the tissues and suppressed the toxic effects of  $\text{Na}^+$ .



**Fig. 5** Effect of *D. tsuruhatensis* DYX29 on ion homeostasis in rice. A, shoot  $\text{Na}^+$  content. B, shoot  $\text{K}^+/\text{Na}^+$  ratio. C, expression level of *OsNHX1*. D, root  $\text{Na}^+$  content. E, root  $\text{K}^+/\text{Na}^+$  ratio. F, expression level of *OsHKT1;1*. Error bars (mean±SD,  $n=3$ ). \* indicates statistical significance (two-tailed Student's *t*-test, \*,  $P\leq 0.05$ ; \*\*,  $P\leq 0.01$ ; \*\*\*,  $P\leq 0.001$ ). Different lowercase letters indicate significant differences (ANOVA, Tukey's HSD test).

**Effect of hormones on the roots of rice** The addition of strain DYX29 significantly increased the contents of IAA, BL, ABA and SA in rice roots by 27.8, 69.5, 123.7 and 28.6%, respectively, under salt stress conditions. Among them, IAA and BL are growth-promoting hormones with important functions in promoting plant growth and development, whereas ABA is regarded as a typical adverse hormone, and SA is also closely related to the plant response to environmental stress. The genes *OsPIN1* and *OsYUCCA1* were involved in the polar transportation and synthesis pathways of IAA, respectively, and the results showed that DYX29 could significantly improve their expression levels.



**Fig. 6** Effect of *D. tsuruhatensis* DYX29 on hormone levels in rice roots. A, IAA content. B, expression level of *OsYUCCA1*. C, expression level of *OsPIN1*. D, BL content. E, ABA content. F, SA content. Error bars (mean±SD, n=3). \* indicates statistical significance (two-tailed Student's *t*-test, \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ).

## 4. Discussion

### 4.1. Analysis of endophytic bacterial communities and identification of key endophytic bacteria in response to salinity

In this study, salt treatments had little effect on the community structure of endophytic bacteria of *K. obovata* propagules and mainly affected some key taxa in the community composition, which is consistent with previous studies. For example, it was found that increasing salinity had no effect on species richness, evenness and  $\beta$ -diversity among natural bacterial communities in freshwater rock pools, suggesting that bacterial communities are functionally and compositionally stable at local scales in response to varying intensities of salinity change (Berga *et al.* 2017). Another study

showed that high salt treatment did not alter the abundance of dominant phyla, including Proteobacteria, Acidobacteriota, and Actinobacteriota in the interroot microbial community of *Matricaria chamomilla* L. while significantly altering the abundance of ecologically relevant functional species, such as *Chytridiomycota* and *Simmonsii* (Xia *et al.* 2023). This phenomenon reflects the existence of relatively stable dominant phyla, and the increase in the abundance of salt-tolerant species and their enhanced ecological functions may be related to the tolerance of host plants to salt stress.

We found that a total of 3 OTUs were significantly different between the salt treatment and the control treatment, which was jointly determined by the salt-tolerance capacity of the bacteria itself and its reaction with the plants in the salt environment. Some researchers have suggested that plants subjected to salt stress may produce specific metabolites to target recruitment or enrichment of specific strains, thus assisting the plants in coadapting to different salt environments (Li *et al.* 2021). The relative abundance of OTU3514 (*Delftia tsuruhatensis*) was significantly and drastically increased after salt treatment. High-throughput data showed that this strain is a key endophytic bacterium in *K. obovata* propagules in response to salinity changes. Currently, HT-PGPB isolated from saline soils mainly includes *Bacillus*, *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Flavobacterium* and *Burkholderia* (Patel *et al.* 2017), while *Delftia tsuruhatensis* has been less reported. This bacterium was first isolated from a domestic sewage treatment plant in Japan (Shigematsu *et al.* 2003). It has been extensively studied in the field of organic degradation and has also been reported to inhibit the quorum-sensing and biofilm formation of certain pathogens (Malešević *et al.* 2019), which stimulates the development of new drugs against antibiotic-resistant bacteria. Studies have shown that this bacterium can secrete siderophores and thus exhibit PGP traits (Hou *et al.* 2015). Our work provides examples and specific mechanistic studies to promote enhanced salt tolerance and growth of crops in saline environments.

The results showed that this strain can survive under high salt conditions and is a high siderophore-producing bacterium. On the one hand, siderophore-producing bacteria can change the effectiveness of iron in the soil through the chelating effect of siderophores, thus improving the iron deficiency in plant rhiza and meeting the nutrient requirements of plant growth. On the other hand, it can indirectly promote plant growth by competing with some pathogenic microorganisms for

iron and inhibiting the growth of the latter (Ghosh *et al.* 2020). In addition, the strain also produces ACC deaminase to hydrolyze ACC, a precursor of ethylene, into  $\alpha$ -butyronic acid and ammonia, thereby effectively reducing the internal ethylene content of the plant under adversity and decreasing the adverse effects of ethylene on plant growth and development. During abiotic stress, ACC deaminase can significantly increase crop resistance and yield. It was reported that PGPRs that produce ACC deaminase can reduce plant growth limits due to water-deficient conditions (Duan *et al.* 2021). Gupta and Pandey (2019) reported that ACC deaminase-producing rhizobacterial strains isolated from the garlic (*Allium sativum*) rhizosphere demonstrated multifarious plant growth-promoting traits and alleviated salinity stress in French bean (*Phaseolus vulgaris*) plants.

Nontargeted metabolomic analyses showed that this strain had the highest number of intracellular metabolites annotated to components of the amino acid metabolic pathway. Glutamate enhances osmoadaptation of anammox bacteria under high salinity, which suggests that glutamate is a major regulator of osmotic stress (Naufal *et al.* 2022). In addition, halophilic bacteria generally have high contents of acidic amino acids, including aspartic acid and glutamic acid, and many proteins enriched in these amino acids have increased solubility and are more resilient in high-salt environments, resulting in better adaptation to stress (Graziano and Merlino 2014). Thus, the enrichment of amino acid metabolic pathways confers salt tolerance to the strain. Additionally, salt treatment induces an increase in amino acids, niacin and 3-indoleacetic acid in the bacterial cytosol. It is well known that amino acids can act as small molecules of cellular osmoprotectants to help the bacterium maintain osmotic balance, which is the basis for the strong salt tolerance of the strain. Niacin (vitamin B3) acts as a coenzyme in the conversion of metabolic hydrogen to NADP<sup>+</sup> as nicotinamide *via* ATP, and the application of niacin treatment promotes plant growth (Wang and Bai 2014; Ali *et al.* 2023). It was reported that the application of nano iron oxide and niacin spraying treatments enhanced nutrient elements and phenolic compounds in *Mentha Piperita* L. (Ali *et al.* 2023). The application of niacin in kiwifruit can enhance the antioxidant capacity of plants (Zhu *et al.* 2018), which demonstrates that niacin can help plants resist stress and promote growth. 3-Indoleacetic acid is a broad-spectrum plant growth regulator with auxin activity, a signaling molecule for organ formation and growth regulation in plants, and it promotes cell division and accelerates rooting, which is the reason why this strain has a growth-promoting

effect on plants in the presence of salt stress.

#### 4.2. *D. tsuruhatensis* DYX29 promotes rice seed germination and rice growth under salt stress

The addition of beneficial microorganisms under salt stress conditions can effectively reduce salt stress and promote plant growth and development. For example, *Arthrobacter* and *Bacillus megaterium* isolated from the interroots of saline plants and inoculated into tomato seeds were found to increase their germination rate, seedling length, vigor index, plant fresh weight and dry weight under salt stress (Fan *et al.* 2016). When soil salinity exceeds 0.3%, rice seedlings exhibit severe symptoms such as browning and wilting of leaf tips and withering of old leaves (Pires *et al.* 2015). The photosynthetic pigment system of plants under salt stress conditions is severely affected, chlorophyll biosynthesis is blocked, and the decomposition process is accelerated. The addition of strain DYX29 in this study effectively maintained the chlorophyll content of rice leaves under salt stress and guaranteed normal photosynthesis in plants. It is noted that the different effects of adding strain DYX29 on the rice seed germination and seedlings may be due to the following reasons: Firstly, in the plate experiment, the growth space for seeds is relatively small, and the cultivation time is short, which limited the rice growth to some extent. Secondly, during the seedling stage, the strain can better colonize in the roots, so it can better promote rice salt tolerance. Similar reports can also be found in literature, where the authors found that the effect of inoculating beneficial microorganisms to control bacterial wilt is better during the early stage of crops (Kaur *et al.* 2022). This may further guide the field practice. It may be possible to achieve better growth-promotion results by appropriately increasing the amount of this strain during the seedling stage of rice.

#### 4.3. Biological mechanism by which *D. tsuruhatensis* DYX29 promotes rice growth under salt stress

The results of this work showed that the addition of DYX29 increased the expression levels of soluble sugar content and betaine synthesis genes in rice, which act as small molecule

osmoregulators in the cell to help the plant carry out normal water metabolism and effectively counteract the threat to osmotic pressure posed by salt stress (Joshi 2022). In addition, when plants are stressed by high concentrations of salt, cell membranes are susceptible to damage, which can lead to large amounts of MDA production, as well as the accumulation of large amounts of reactive oxygen species. However, there are several enzymatic defense systems in plants, including enzymes such as SOD, CAT and POD, which act synergistically within the cell to control the amount of free radicals in the plant, thus mitigating the damage that occurs to the cell membrane system (Challabathula *et al.* 2022; Guo *et al.* 2022). In the present study, under both H<sub>2</sub>O treatment and 150 mmol L<sup>-1</sup> NaCl treatment, the addition of strain DYX29 reduced the malondialdehyde content in cells and increased the activity of three protective enzymes, i.e., SOD, CAT and POD, which increased the rate of scavenging of intracellular reactive oxygen species, thus jointly protecting the morphology and normal functions of the cell membrane. At the same time, DYX29 can help rice maintain ionic homeostasis in tissues, improve Na<sup>+</sup> exclusion and K<sup>+</sup> uptake, and increase the K<sup>+</sup>/Na<sup>+</sup> ratio, thus more directly reducing the toxic effects caused by Na<sup>+</sup> in leaves and roots.

Strain DYX29 can produce IAA. PGPR have been reported to enhance the salt tolerance of host plants by altering their endogenous hormone status. It was reported that *Myroides* sp. JIL321 isolated from the soil of the rice rhizosphere with high IAA production improved the growth of salt-stressed rice seedlings (Wang *et al.* 2022). Other researchers have suggested that the increase in IAA concentration increases plant salt tolerance by regulating ion homeostasis in plant tissues and microbial communities in the rhizosphere soil and stimulating root proliferation (Yu *et al.* 2020; Feng *et al.* 2023). It has also been reported that endogenous hormones, including IAA, could improve the salt tolerance of maize (*Zea mays* L.) by inducing root architecture and ion balance optimizations (Hu *et al.* 2022). The above-mentioned studies are in accordance with the results of the present study, suggesting that IAA synthesized by strains induced by high salt is directly related to seed adaptation to salt stress. Salt stress may directly regulate BR signaling at the transcriptional and posttranslational levels (Yu *et al.* 2020), and some key enzymes involved in BR synthesis are needed for plant salt acclimation, suggesting that BRs make plants salt tolerant (Tanveer *et al.* 2018). ABA maintains the hydraulic conductivity of stems and roots, which allows the plant to make better use of soil water while maintaining water absorption by upregulating the

antioxidant system and osmoregulatory capacity (Golldack *et al.* 2014). In general, it can be concluded from these studies that growth-promoting hormones and adversity hormones tend to exhibit antagonistic effects, while there are also studies that support their collaboration. For example, synergistic interactions between ABA and BRs are clearly present at specific stages, including seedling leaf development and early low concentrations of ABA-mediated salt stress tolerance, through a molecular mechanism dependent on the ABI3-OsGSR1 module (Waadt *et al.* 2022). Meanwhile, mild salt stress induces a small amount of ABA, which activates plant growth hormone signaling, thereby forming the lateral root primordium (LRP), i.e., inducing the development of lateral roots to acclimatize to the environment (Ding *et al.* 2015). SA is another important phytohormone, and previous studies indicate that SA promotes Na<sup>+</sup> efficient segregation and partitioning (Jayakannan *et al.* 2015) and higher photosynthetic rates and antioxidant enzyme activities (Ahanger *et al.* 2019), which are in line with the trends of the data obtained in this study. Overall, the addition of strain DYX29 can synthesize and release exogenous stimulatory signals such as plant hormones and alter the endogenous hormone status of rice during salt stress. The plant hosts also finely coordinate the signals from endogenous and exogenous hormones, balance the effects of different hormones, and then adapt to adversity.

## 5. Conclusion

In this work, the diversity of the endophytic bacterial community in the propagules of *K. obovata* under salt treatment was analyzed, and *D. tsuruhatensis* DYX29, a key species that responds to changes in salinity, was identified and screened. Combined with nontargeted metabolomic analysis, it was demonstrated that the acquired key strain DYX29 can maintain intracellular osmotic pressure through amino acid metabolism and the ability to synthesize phytoconductive substances. Inoculation with strain DYX29 significantly promoted rice seed germination and seedling biomass accumulation under salt stress. The analysis of the intrinsic mechanism of salt tolerance of this bacterium was accomplished in terms of the regulation of four aspects: osmotic capacity, antioxidant system, ionic homeostasis, and hormone levels in rice tissues. It is concluded that the vertically transmitted endophytic bacterium *D. tsuruhatensis*, which responds to salinity changes in

*K. obovata*, can help rice resist salt stress and may provide new biomaterials for the biological management of saline soils. This work provides a research paradigm for anchoring key species by analyzing changes in environmental microbial communities and carrying out *in vitro* validation and application of their functions. Subsequently, further studies on the effect of this bacterium on salt tolerance in rice and its microecological mechanism in salt-stressed experimental fields can be carried out. In prospect, it may provide a basis for the use of probiotics based on salt-tolerant bacteria to increase crop yield in agricultural fields, especially in saline-alkali land.

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### **Declaration of competing interest**

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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