A bacterial formulation based on two exopolysaccharide-producing rhizobacteria from Dongxiang wild rice (Oryza rufipogon Griff.) confers drought tolerance in cultivated rice Dongdao-4 (Oryza sativa L.)

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Research Article

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Abstract

Objectives.

The present study aims to isolate exopolysaccharide-producing bacterial strains from the rhizosphere of Dongxiang wild rice (*Oryza rufipogon* Griff.); elaborate a bacterial formulation, and quantify its effect on the defense against stress in cultivated rice seedlings under drought.

Methods.

Dongxiang wild rice rhizospheric soil was used to isolate exopolysaccharide-producing bacteria; and bacteria isolates were identified, at the taxonomic level of genus, following polyphasic methods. A bacterial formulation was made; and the enzymatic activity and the malondialdehyde content were quantified; in addition to measuring morphological indicators of growth in cultivated rice Dongdao-4 (*Oryza sativa* L.) seedlings under drought stress.

Results.

The inoculation of cultivated rice with the bacterial formulation made from two selected isolates had positive impacts on growth parameters and the antioxidant defense under drought, significantly surpassing the effect of the commercial products PB (Biofertilizer P) and EM (Efficient Microorganisms). Even the shoot length of the inoculated plants under drought does not differ significantly from control plants under normal water conditions; while the root dry weight was significantly higher. The enzymatic activity of the inoculated plants significantly exceeds the other treatments in drought; and the malondialdehyde content was the lowest of the treatments. The exopolysaccharides-producing bacterial strains mitigate the adverse effects of drought stress; and the bacterial formulation improve relevant parameters of the plants under drought, and can be used as a potential inoculant in arid zones.

Conclusions.

This study demonstrates the efficacy of exopolysaccharide-producing Dongxiang wild rice rhizobacteria in improving drought tolerance and consequently enhancing plant growth.

Introduction

Sustainable agricultural practices, such as the use of plant growth promoting rhizobacteria (PGPR) or microbial inoculants, are gaining prominence in intensive agriculture worldwide because the use of agrochemicals in crop production is being reduced due to the environmental and economic problems that come with it (Anli et al. 2021; Atieno et al. 2020; Basu et al. 2021).

PGPRs can facilitate plant growth by direct and indirect mechanisms that occur inside and outside the plant, respectively (Backer et al. 2018; De la Fuente et al. 2020). Direct mechanisms include providing phytonutrients to plants such as fixed nitrogen or solubilized soil minerals (such as phosphorus,

potassium and iron) and stimulating plant growth and development by regulating phytohormone levels (Gouda et al. 2018; Kalam et al. 2020). Indirect mechanisms include influencing plant health by suppressing plant pathogens and other harmful microorganisms, competing for nutrients and producing antagonistic substances such as hydrogen cyanide and siderophores, and inducing systemic resistance in plants against a broad spectrum of root and leaf pathogens (Berg et al. 2017; Meena et al. 2020; Sayyed et al. 2019).

The PGPR also enhances tolerance to various abiotic stresses in plants and some of the efficient mechanisms under abiotic stress include the production of stress-related hormones, osmolytes, antioxidant enzymes, and exopolysaccharides (Ilyas et al. 2020).

Abiotic stress factors include heat, salinity, chilling, nutrient deficiency, and drought. As a consequence of global warming and soil degradation, drought is becoming one of the main challenges for agriculture productivity in arid and semiarid regions (Kompas et al. 2018). Drought stress has a serious effect on important crops such as pea, alfalfa, and rice.

Rice is the second major staple crop for human as half of the global population depends on it to meet their daily calorie requirement (Ahmad et al. 2019; FAO 2020; Hussain et al. 2022). Drought stress negatively affects the growth and productivity of the crop (Shekoofa and Sinclair 2018). The morphological and physiological functioning in rice plants changes drastically due to drought (Abdou et al. 2021; Yang et al. 2019), such is the case of root length density, root moisture extraction, the rate of apical development, leaf senescence, leaf elongation, dry matter production, malondialdehyde content (MDA) as a final product of lipid peroxidation, proline content, soluble sugar content, protein content, photosynthesis and antioxidant defense (Yang et al. 2019).

Previous studies revealed that Dongxiang wild rice (DXWR, *Oryza rufipogon* Griff.), from Jiangxi province, China, possesses high resistance to diseases and insects, as well as tolerance to cold and drought, suggesting that it is a valuable research resource for the improvement of different stresses resistance in cultivated rice (Li et al. 2019; Qi et al. 2020). Zhou et al. 2016 found that compared to cultivated rice, DXWR had a very high survival rate under salt stress. Microorganisms associated with wild rice plants may play an important role in affecting significant plant properties (Zhang et al. 2021); some of these microorganisms can enhance plant resistance to stress through interaction with the host plant (Tian et al. 2017).

One study revealed an endophytic bacterium from wild rice germplasm, *Microbacterium laevaniformans*, with a high production of indoleacetic acid (IAA) and gibberellic acid (Borah et al. 2021), and these characteristics have been associated with drought tolerance. Another characteristic that is strongly associated with drought tolerance is the production of exopolysaccharides (EPS). This macromolecule has unique water retention and cementing properties and therefore plays a vital role in regulating the flow of water and nutrients through plant roots for the formation of biofilms (Nivitha et al. 2019).

Recent studies reported that EPS-producing bacteria significantly improve the growth and yield of mung beans (Uzma et al. 2022), rice (Zhang et al. 2020) and tomato (Ghosh et al. 2019) under conditions of drought stress. According to Saikia et al., 2018, the GAP-P45 strain of *Pseudomonas putida*, which has the ability to produce EPS, alleviates drought stress in sunflower (*Helianthus annuus* L.) seedlings by activating the antioxidant enzymatic machinery of the host plant.

EPS-producing bacteria improve soil aggregation and protect plants under water scarcity by retaining a higher water potential around the root area (Ojuederie et al. 2019). Therefore, it is expected that the production of EPS may allow a microenvironment that absorbs water and dries out more gradually than the neighboring microenvironment, thus defending bacteria and plants from drought and fluctuations in water potential, protecting the cell membrane of plants, which in turn favors their normal growth and development.

The objective of this study was to isolate bacterial strains from the rhizosphere of Dongxiang wild rice that show tolerance to drought through the production of EPS; evaluate other plant growth promoting activities of the strains; prepare a bacterial formulation; and to quantify its effect on the defense against stress in cultivated rice seedlings under drought, either through the enzymatic activity and the MDA content of the plant, as well as morphological indicators of growth.

Materials and methods

Isolation and drought screening

This study was carried out in a greenhouse belonging to the Faculty of Life Sciences, Jilin Agricultural University, China, during February 2021. The soil used was black soil (classified as dark Chernozems or Mollisols by the World Reference Base classification system). The main characteristics of the soil were the following: pH = 5.6, electrical conductivity = 0.21ms.cm^{-1}, organic matter = 31.4 g kg^{-1}, available nitrogen = 114.5 mg kg^{-1}, available phosphorus = 19.02 mg kg^{-1}, and available potassium = 14.3 mg kg^{-1}.

We use DXWR; and before sterilizing the seeds, a shelling method was adopted to increase the germination rate around 40% according to Xu et al. (2016). After seven days of germination, seeds were placed in pots and cultivated under normal conditions (28°C, RH 70% and 16 h light) for 90 days. Subsequently, a drought stress treatment was applied, suspending irrigation for 20 days. Finally, a 50 g portion of rhizosphere soil was taken for each plant. The samples were stored in sterile plastic bags and kept in the refrigerator until used.

In the laboratory, for each rhizosphere soil sample, one gram, taken using a quartering method (Campos and Campos 2017), was weighed into 250 ml Erlenmeyer flasks containing 100 mL of Luria-Bertani (LB) culture medium (Valle et al. 2022) and with a PEG 6000 concentration of 43.8% to achieve an osmotic potential of -2MPa (Aujla and Paulitz 2017). It was incubated for 7 days at 28°C, 120 rpm; then serial

dilutions were made at 1 mL scale in sterile saline solution (0.85% NaCl) in order of 10 to 10^{-5}; and 0.1 mL of the appropriate sample dilutions were plated on LB agar and incubated for 48 h at 28°C. Bacterial colonies were chosen based on their colony morphology, further purified and maintained on slants at 4°C and in 65% glycerol at -80°C until further use.

Determination of the EPS production

The bacterial isolates were inoculated into 100 mL of Trypticase Soy Broth (TSB) (Arun et al. 2020) with PEG 6000 (-2 MPa), and incubated for 3 days in a rotary shaker at 28°C, 120 rpm. The cultures were first centrifuged at 10,000 rpm for 10 min to obtain a cell-free supernatant. The soluble EPS in the supernatant was precipitated with two volumes of prechilled acetone and further extracted by centrifugation at 12,000 rpm for 20 min. The analytical method used was the Phenol-sulfuric acid method by Dubois et al. (1956). The precipitated EPS was subsequently dissolved in distilled water and added to a mixture of 1 mL of 5% (w/v) phenol solution and 2 mL of EPS solution and thoroughly mixed. To the reaction mixture, 5 mL of concentrated H_2SO_4 was added and the absorbance was measured at 490 nm. The EPS content was determined from the calibration curve using glucose as the standard (López-Legarda et al. 2017).

Detection of plant growth-promoting traits

For the determination of IAA, the cultures were inoculated into modified Luria-Bertani (LB) liquid medium which contains: L- tryptophan: 10 g; yeast extract: 5 g; NaCl: 10 g, distilled water: 1000 mL; and PEG 6000 (-2 MPa), sterilized at 121°C for 20 min. The cultures were incubated on an orbital shaker for 7 days at 28°C at 120 rpm. IAA production was evaluated by the colorimetric method; 5 mL bacterial culture was centrifuged for 10 min at 8000 rpm, and 2 mL of the supernatant was added with 4 mL of Salkowski's reagent (0.5 mol L^{-1} $FeCl_3$ in 35% $HClO_4$). The uninoculated medium was used as a control. Pink color development with the addition of Salkowski's reagent and cell-free culture supernatant (4:2) indicated the formation of indole compounds, and after incubation for 30 min, the optical density of the supernatant was measured in the spectrophotometer at 530 nm. IAA concentration was determined with a standard curve of pure indole-3-acetic acid (IAA, Hi-mean) ranging from 0 to 50 µg mL^{-1} (Gusmiaty et al. 2019).

Phosphorus solubilization (P), 100 mL of NBRIP broth (Valle et al. 2022) with PEG 6000 (-2 MPa) were inoculated with 1 mL of bacterial cultures (10^8 CFU mL^{-1}). The cultures were kept at 28°C for 12 days in an incubator shaker at 120 rpm. Cells were harvested by centrifugation at 10 000 rpm, for 10 min. The supernatant was assayed for the soluble phosphorus concentration and anti-molybdenum-antimony dye method was used (Li et al. 2020).

For ammonium production (NH_4^+) was carried out using peptone water broth as per the methodology of Di-Benedetto et al. (2019) only under the nonstress condition; 100 mL of peptone water broth were inoculated with 1 mL of bacterial culture (10^8 CFU mL^{-1}), the cultures were kept at 28°C for 8 days in an incubator shaker at 120 rpm. Cells were collected by centrifugation at 10 000 rpm, for 10 min. Nessler's reagent was added (1 mL/tube), and development of yellow to the brown color indicated NH_4^+ production.

For qualitative potassium solubilization (K) was used Aleksandrov agar medium (Valle et al. 2022), on the basis of the area of solubilization only under the nonstress condition. If a transparent halo around the colony is evidenced, it will be considered an indicator for potassium dissolution. The inoculated plates were placed in the incubator at 28°C for 7 days until the growth of the isolates were observed.

The qualitative estimation of ACC deaminase was carried out following the procedure of Penrose and Glick (2003). The isolates were inoculated into 100 mL of the sterile minimal DF (Dworkin and Foster 1958) salts media with PEG 6000 (-2 MPa) amended with 3 mM ACC as the nitrogen source, and incubated on a rotary shaker at 120 rpm, and 28°C for 48 h. Bacterial fluid in was plated onto solid DF salts agar medium containing ACC (500 µmol mL^{-1}), and incubated for 3–4 days at 28°C. The colonies that grew indicated the ACC deaminase activity (Mir et al. 2022).

Siderophores were detected following Mir et al. 2022 by the formation of orange halos around bacterial colonies on Blue Agar CAS (Louden et al. 2011) after incubation for 4 days at 28°C only under the nonstress condition.

Production of Hydrogen cyanide (HCN) was qualitatively determined by the method of Bakker and Schippers (1987) only under the nonstress condition. The strains were grown in King’s solid medium modified with 4.4 g of glycine (King et al. 1954). The plates were incubated at 28°C for up to 4 days. The positive production of HCN was visually evaluated according to the change in the color of the filter paper from yellow to dark brown, depending on the intensity of secretion.

Identification of bacteria

Colony morphology, size, and Gram stain of the bacterial isolates were examined according to (Christopher and Bruno 2003). Individual cultures grown on LB agar medium at 28°C were examined for the colony features, and Gram staining was performed with exponentially growing cultures.

Sequencing of 16S rRNA gene

The pure colony of the isolates were sent to Jilin Kumei Biotechnology Co., Ltd, China, where the 16S rRNA gene was bidirectionally sequenced according to universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT − 3') (Sharp et al. 2006), using an ABI 3730xl sequencer by the Sanger method. The sequences were constructed with Vector NTI Advance software version 8 (version 8.0; Informax Inc., Bethesda, Md.) and compared to the NCBI database using BLASTN 2.6.0 + software and the MEGA6 algorithm (Tamura et al. 2013). The sequences were aligned using the CLUSTAL-X algorithm (Thompson et al. 1994), and the identity values were calculated from the pairwise distance estimate. The partial 16S rRNA gene sequences were deposited in GenBank data base (http://www.ncbi.nlm.nih.gov/).

Phylogenetic trees were constructed using the Maximum Likelihood (ML) method (Tamura et al. 2013) and the heuristic methods of nearest neighbor exchange, Kimura's two-parameter model, uniform

velocities and complete deletion were chosen (Kimura 1980). The robustness of the reconstructed phylogenetic trees was confirmed by Bootstrap analysis based on 1000 replicates.

Antagonism compatibility test

An antagonism test was carried out for the compatibility test between the isolates. The combinations between bacteria were determined; 0.1 mL of the bacterial suspension was inoculated opposite each other at a distance of 2 cm on nutrient agar medium and incubated at 28°C for 5–7 days. The experiment was performed in triplicate. The appearance of the zone of inhibition between the isolates indicate the antagonistic activity (Widyantoro et al. 2019).

Evaluation of the bacterial formulation in drought conditions

Experimental materials

We used the cultivated rice variety Dongdao-4, previously identified as an alkali-tolerant cultivar grown in western Jilin province, China (Lin et al. 2022). First, the seeds were sterilized and placed to germinate in Petri dishes according to the procedure of Valle et al. (2022). Then, uniform seedlings were randomly sown on a floating tray system with black soil sterilized in an oven (80°C for 3 h), and autoclaved tap water (121°C, 1013 hPa for 20 min). After two weeks, the rice seedlings were transplanted into 1 kg pots with black soil without any fertilizer. The pot culture experiment was carried out in a completely randomized system of 5 treatments and three replicates, with each pot consisting of four plants.

Experimental treatments

The treatments began at the time of transplanting starting from the same soil moisture conditions (at field capacity). Drought stress was imposed by water retention for 10 days, and then all pots were watered to saturation on day 11. Control positive plants were constantly watered at 2-day intervals. Autoclaved tap water was used. After 2 days of recovering (27 days of seedling age) relevant parameters were measured.

Treatment details are as follows: (1) CK(-)WL (drought + foliar application of sterile water); (2) Chosen bacteria (drought + foliar application of the bacterial formulation), (3) PB (drought + soil inoculation of the commercial product); (4) EM (drought + soil inoculation of the commercial product); (5) CK(+) (plants under normal water conditions).

Bacterial application and drought were imposed during the vegetative stage of Dongdao-4 rice plants, as it is a sensitive phase to water deficit conditions. Bacterial application was carried out on the first and seventh day after the start of the drought treatment (Arun et al. 2020); and 10 mL of the treatment (2) was sprayed twice with an interval of 1 hour to guarantee absorption by the seedlings, with a final dose of 20 mL for each pot. The entire experimental setup was established under greenhouse conditions (28°C, RH 70% and 16 h light).

Preparation of the bacterial formulation (Treatment 2)

To prepare the bacterial formulation the strains grew in 100 mL of LB medium in Erlenmeyer flasks, incubated at 28°C at 120 rpm until late exponential phase and centrifuged at 10 000 rpm for 10 min at 4°C, washed and resuspended in 400 mL of sterile autoclaved tap water and adjusted to 10^8 CFU mL^{-1} through viable counts, and the experimental combination of the strains was made (50:50 mL) for a final volume of 100 mL.

Preparation of the commercial products (Treatments 3 and 4)

The commercial products PB (Biofertilizer P) and EM (Efficient Microorganisms, Sanko Sangyo Co., Ltd.) were prepared as described in http://www.gzwybio.com/se_show_16.html for PB, and https://saion-em.co.jp/ for EM; and 20 mL of each one were added to the soil at a concentration of 10^8 CFU mL^{-1} two days before transplant.

Relevant parameters in rice treated with the bacterial formulation in drought conditions.

Morphological parameters

The seedlings were carefully uprooted and washed with tap water. Root/shoot lengths were measured with a millimeter ruler; and the fresh plant biomass and the dry biomass of the seedlings were determined by drying in an oven at 80°C until a constant weight was obtained. Weights were recorded with a digital scale.

MDA content

MDA content was calculated in μmol L^{-1} FW by the method of Del Buono et al. (2011) with some modifications. Briefly, homogenate from leaves (0.5 g, FW) ground in the presence of 5% (w/v) trichloroacetic acid (TCA) was centrifuged at 12 000 g for 15 min. Then, the supernatant was mixed with 5 mL of 0.5% thiobarbituric acid (TBA), prepared with 20% TCA, and mixture was incubated 100°C for 25 min. Next, the reaction liquid was cooled to room temperature and centrifuged at 7 500 g for 5 min. Finally, the absorbance of the supernatant was measured at 450, 532, and 600 nm, and the MDA content was calculated by the following formula: C_{MDA} (μmol L^{-1}) = C (μmol L^{-1}) x V (L)/ fresh weight (g) x 1000, where C (μmol L^{-1}) = 6.45 (A_{532} – A_{600}) – 0.56 A_{450}, and V refer to the volume (L) of extracting solution.

Antioxidant defense

Leaves (0.5 g, FW) were homogenized in 1.5 mL of pre-chilled Tris-HCl buffer (pH 7.5) containing 5% sucrose and 0.1% mercaptoethanol. The homogenate was centrifuged at 10,000 g for 20 min at 4°C (Sun et al. 2020b). Then, the supernatant was used as a crude enzyme extract to determine the activities of SOD, CAT, and POD following Lei et al. (2015).

Superoxide dismutase activity (SOD), the reaction system contained 1.5 mL of 0.05 M phosphate buffer, 0.3 mL of 130 mM methionine solution, 0.3 mL of 750 μM nitroblue tetrazolium (NBT) solution, 0.3 mL of 100 μM EDTA-Na_2 solution, 0.3 mL of 20 μM lactochrome solution, 0.5 mL of distilled water, and 0.1 mL of crude enzyme extract. The reaction was started and maintained under 4000 lx of light for 20 min. A reaction, in which the crude enzyme extract was replaced with a phosphate buffer under the same light intensity for 20 min, was used as a control. Another reaction, which contained phosphate buffer and was incubated in the dark for 20 min was used as a blank. At the end of the reaction, the absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibited NBT reduction by 50%, and the results are expressed as units per mg of protein.

Catalase activity (CAT), the reaction system contained 0.1 mL of 2% H_2O_2 and 2 mL of 50 mM phosphate buffer (pH 7.0), and the reaction was started by adding 0.1 mL of crude enzyme extract. At the end of the reaction, the absorbance was measured at 240 nm. CAT activity is reported as the decrease in H_2O_2 per minute and was calculated using the following formula:

$$CAT = \frac{\Delta A240 \times Vt}{0.1 \times V1 \times t \times Fw},$$

where V refer to the volume (L), and Fw refer to fresh weight (g).

Peroxidase activity (POD), the reaction system contained 2.9 mL of 0.05 M phosphate buffer, 0.5 mL of 2% H_2O_2, 0.1 mL of 2% guaiacol, and 0.1 mL of crude enzyme extract. At the end of the reaction, the absorbance was measured at 470 nm. POD activity was defined as the amount of guaiacol oxidized per minute.

Statistical Analysis

All the experiments were conducted with a minimum of three replicates, and the results were analyzed with Origin 8 and expressed as the mean ± standard deviation. For the statistical analysis, using IBM SPSS Statistics Ver. 21 software, One-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple range test ($p \leq 0.05$). For the NH_4^+ production samples, an Independent-Samples T-Test ($p \leq 0.05$) was used.

Results

Isolates tolerant to desiccation

Thirteen isolates were obtained that could grow in LB broth at an osmotic potential of -2MPa (PEG 6000), and as a result of a subsequent selection, based on the EPS production, only the isolates N1g and N2 had values more than 50% higher than the rest of the isolates, so they were chosen as promising isolates. For both isolates, under stress conditions, EPS production is significantly affected compared to EPS

production without stress, showing from 950.4 µg glucose mL^{-1} to 764.8 µg glucose mL^{-1} for N1g, with a reduction of 24.5%; and for N2 it was shown from 961.7 µg glucose mL^{-1} to 723.8 µg glucose mL^{-1}, with a reduction of 32.9% (Table 1).

Plant growth-promoting traits by bacterial isolates

IAA production values are shown in Table 1; and the highest IAA production under non-stressed conditions is observed for N1g (743.7 mg L^{-1}), which is significantly affected under stress conditions (81.3 mg L^{-1}) and does not differ significantly from N2 under the same stress with a production of 101.8 mg L^{-1}.

The phosphorus solubilizing capacity assay revealed that under non-stressed conditions N2 significantly exceeds N1g with 468.5 mg L^{-1} as is shown in Table 1; while under stress conditions, the phosphorus solubilizing capacity decreased significantly to 5.73 mg L^{-1}(N1g) and 4.54 mg L^{-1}(N2), without significant differences between both isolates.

Table 1 Plant growth promoting properties of N1g and N2 under non-stressed conditions and drought-stressed conditions.

	Isolates			
Plant growth promoting properties	N1g		N2	
	Non-stressed conditions	Drought-stressed conditions	Non-stressed conditions	Drought-stressed conditions
EPS production (µg glucose mL^{-1})	950.4 ± 53.9 a	764.8 ± 56.9 b	961.7 ± 39.4 a	723.8 ± 1.2 b
IAA production ($mg\ L^{-1}$)	743.7 ± 8.2 a	81.3 ± 0.85 c	596.9 ± 7.1 b	101.8 ± 3.5 c
P-solubilization ($mg\ L^{-1}$)	194.9 ± 3.61 b	5.73 ± 0.28 c	468.5 ± 38.4 a	4.54 ± 0.28 c
NH_4^+ production ($mg\ L^{-1}$)	666.2 ± 43.1 a	ND	503.8 ± 4.5 b	ND
ACC deaminase activity	+++	++	++	+
Potassium solubilization	-	ND	+++	ND
HCN production	-	ND	++	ND
Siderophores production	++	ND	+++	ND

The ACC deaminase activity was shown to be excellent (+++) for N1g under non-stress conditions, and under drought-stressed conditions showed good activity (++). While for N2, under non-stress conditions, the ACC deaminase activity was shown to be good (++); and under drought-stressed conditions was observed present (+) (Table 1).

For NH_4^+ production, N1g showed a significantly higher production with 666.2 mg L^{-1}; and the potassium solubilization and HCN production it was only positive for N2 (Table 1). Siderophore production showed excellent activity (+++) for N2, and good activity (++) for N1g (Table 1).

Drought-stressed conditions: PEG 6000 (-2 MPa). Values are the mean ± standard deviation of experimental data in triplicate. Letters: a; b; c is the comparison between bacteria. Symbols: +, presence; ++, good; +++, excellent; -, no presence. ND undetermined. Values with different letters and symbols are significantly different according to Tukey's multiple range test ($p \leq 0.05$). For the NH_4^+ production samples, an Independent-Samples T-Test ($p \leq 0.05$) was used.

Phenotypical, biochemical and molecular characterization of the bacterial isolates

Both isolates showed catalase activity, were positive for methyl red test, did not use citrate and were negative in the "Voges Proskauer" test (they did not produce acetoin from glucose), hydrolyzed starch and were negative for the indole test. Both isolates fermented glucose and sucrose, and did not ferment lactose.

Microscopic studies revealed isolates N1g and N2 as Gram-positive and Gram-negative respectively; and after growing seven days on LB agar both isolates have a large colony size, a cream color, flatted elevation, smooth shiny surface, opaque density, and membranous consistency. A rhizoid shape for N1g, and circular for N2, a lobed edge for N1g, and an entire edge for N2.

The 16S rRNA gene sequence obtained for the isolates N1g (1 437 bp) and N2 (1 428 bp) were deposited in GenBank under accession number ON409576 and ON409577 respectively. In the constructed phylogenetic trees it can be seen that the isolates N1g (Fig. 1a) and N2 (Fig. 1b) it is located within the phylogenetic radiation of the *Bacillus* and *Enterobacter* genus respectively.

Pairwise comparisons indicated that isolate N1g had high similarities to *Bacillus thuringiensis* strain IAM 12077T (100%), *Bacillus cereus biovartoyoi* strain NCIB 40112 (100%) and *Bacillus cereus* strain ATCC 14579T (99.8%). N2 had high similarities to *Enterobacter roggenkampii* strain DSM 16690 (99.6%), *Enterobacter tabaci* strain YIM Hb-3 (99.4%) and *Enterobacter asburiae* strain JM-458 (99.1%).

The antagonism test between the bacteria indicated that there was no antagonistic activity between the strains.

Relevant parameters in rice treated with N1g+N2 formulation in drought conditions.

Drought stress drastically affected the growth of shoots and roots, and the drying of leaves by 100% for the CK(-) WL and PB groups, while for the EM group it was affected by 80%. The N1g+N2 group, named in Materials and methods as treatment 2, is only affected by 10%, with 90% of plants recovered after post-stress recovery irrigation (Fig. 2).

Morphological parameters

As shows Fig. 3a, data on shoot length indicate that inoculation with N1g+N2 enhanced shoot length significantly (P = 0.05) in comparison with the negative control CK(-)WL, being 24.5% higher. The shoot length with the N1g+N2 treatment was the largest reaching 28.6 cm, and also differed significantly from the commercial biofertilizers PB and EM, with increases of 33.2% and 17.3% respectively. In root length (Fig. 3a), inoculation with N1g+N2 treatment do not differ significantly from the inoculation with biofertilizers PB and EM, and these three treatments do not differ significantly from the non-inoculated group CK(-)WL.

Inoculation with N1g+N2 treatment showed significant (P = 0.05) increase in shoot fresh weight (Fig. 3b) as compared to uninoculated control CK(-)WL, with an increase of 199.1%. The treatment N1g+N2 also outperforms biofertilizers PB and EM, with increases of 216.3% and 128.4% respectively. The maximum

root fresh weight (178% over uninoculated control CK(-)WL was observed in N1g+N2 treatment (Fig. 3b); this treatment also showed a significant (P = 0.05) increase of 42.9% over healthy plants control CK(+) and increased the root fresh weight over biofertilizers PB and EM by 158.4% and 72% respectively.

The results for shoot dry weight (Fig. 3c) showed that N1g+N2 treatment significantly (P = 0.05) increased the shoot dry weight in comparison to uninoculated control CK(-)WL by 65.9%. N1g+N2 treatment also outperforms biofertilizers PB and EM with increases of 108.8% and 45.7% respectively. Root dry weight of rice seedlings inoculated with N1g+N2 significantly outperform CK(+), CK(-)WL, and PB with increases of 34.1%, 48.8%, and 72.1% respectively (Fig. 3c).

MDA content

The lower concentration of MDA was reflected in the seedlings treated with the bacteria (N1g+N2), significantly reduced (P = 0.05) compared to the negative control (69.5%) and the commercial products PB (39%) and EM (15.7%) (Fig. 4a).

Antioxidant defense

The analysis of the antioxidant activity of the enzymes in the plants under drought stress treatment showed that the application of the bacterial formulation improved the response to stress. SOD activity in seedlings inoculated with N1g+N2 significantly (P = 0.05) increased over the uninoculated control CK(-)WL, PB and EM, with increases of 22.3%, 17.6% and 17.4% respectively. The PB, EM and CK(-)WL treatments do not differ from each other (Fig. 4b).

Highest CAT activity was observed in the seedlings inoculated with N1g+N2 (304.8 % over uninoculated control CK(-)WL), showing significant increases over biofertilizers PB and EM by 362% and 224.4% respectively. PB, EM and CK(-)WL treatments do not differ from each other (Fig. 4c). The results for POD activity (Fig. 4d) showed that N1g+N2 treatment significantly (P = 0.05) increased POD activity in comparison to uninoculated control CK(-)WL, PB, and EM with increases of 185.7%, 129.9%, and 25% respectively.

Discussion

Environmental factors play an important role in the evolution of plant species (Sun et al. 2020b). However, many studies have found that microorganisms are also important in the evolution of host plant species. These microorganisms can improve the resistance of plants to stress through interaction with the host plant, especially in wild species. The study and detection of culturable bacteria of wild rice is important, since some bacteria can influence desirable characteristics of resistance to environmental factors such as drought (Tian et al. 2017). The drought tolerance of DXWR has been previously reported; as well the potential of DXWR-associated bacteria to promote plant growth, among which is the genus *Bacillus* spp. (Zhang et al. 2021) and *Enterobacter* spp. (Ahmed et al. 2021).

This study demonstrates the effectiveness of exopolyscaride-producing rhizobacteria associated with DXWR in inducing drought tolerance and consequently enhancing rice plant growth under drought stress conditions. The length of the sequenced 16S amplicons from the N1g and N2 isolates was sufficient for the subsequent phylogenetic studies, as the minimum required length is of 500 bp – 525 bp, with a recommended amplicon size of 1 300 bp to 1 500 bp for this gene (Janda and Abbott, 2007; Bonatsou et al. 2019). The sequencing results enabled the placing of isolate N1g in the phylogeny of the genus *Bacillus*. On the other hand, the sequences obtained for N2 allow us to locate this isolate within the *Enterobacter* genus.

These strains obtained, whose genera have already been reported in tolerance studies to different types of stress, provide a novel characteristic for drought tolerance studies, which is the ability to produce exopolyscarides (EPS) in the first place, in addition to the other potentialities of plant growth promoting such as the production of indole-3 acetic acid (IAA), phosphate solubilization (P), NH_4^+ production, 1-aminocyclopropane deaminase activity-1-carboxylate (ACC deaminase), potassium solubilization, HCN production, and production of siderophores. Both strains show a high production of these abilities under non-stress conditions; but in drought-stressed conditions using PEG 6000 at an osmotic potential of -2 MPa, they still show a very potent ability to produce EPS and IAA.

The inoculation of EPS-producing strains is a key factor for improving plant growth under drought stress (Ilyas et al. 2020; Khan et al. 2019). EPS are hydrated compounds with 97% water in the matrix that protects both bacteria and plants from drying out. It has been reported that a wide variety of EPS-producing rhizobacteria help crop plants to better stress tolerance (Etesami and Adl 2020), as they improve water retention by maintaining diffusion from organic carbon sources (Yasmin et al. 2020). Plants inoculated with EPS-producing bacterial strains are more drought tolerant because these strains maintain the stability of soil aggregates and retain water content, which in turn increases plant growth (Asghari et al. 2020).

Ahmed et al. 2021 evaluated the impact of water stress on EPS production by PAB19; and obtained that EPS production was reduced in a dependent manner by the PEG-6000 dose. In non-stressed controls PAB19 produced a considerable amount (455.3 µg mL^{-1}) of EPS, but it decreased as water stress increased up to 204 µg mL^{-1} at a concentration of 15% PEG-6000. While in our study, a higher amount of EPS was obtained in the controls without stress, with 950.4 µg mL^{-1} for N1g and 961.7 µg mL^{-1} for N2; and even under severe stress conditions (43.8% PEG-6000) high levels of EPS production were maintained, with 764.8 µg mL^{-1} for N1g and 723.8 µg mL^{-1} for N2, which represents increases of 275% (N1g) and 254% (N2) with respect to the EPS production of the PAB19 strain from the study by Ahmed et al. 2021 under less stress conditions (15% PEG-6000). Our strains, even under severe water stress conditions, show increases of 68% and 59% compared to the EPS production of the PAB19 strain under non-stress conditions.

Thus, the production of EPS at high PEG concentrations is a clear indication that our N1g and N2 strains can withstand severe drought stress; and drought tolerance can protect bacterial cell development and

improve survival and activity under water stress, to in turn protect the physiological processes that help the development and defense of the plant.

IAA is a physiologically active auxin (Patten and Glick 1996), which regulates growth and various physiological and biochemical processes of plants even under drought stress conditions (Deinum et al. 2016); and recently, the production of IAA has been proposed as one of the main characteristics to identify in the new PGPM (Ali et al. 2022).

Panigrahi et al. 2020 have reported that strain OS03, identified as *Enterobacter cloacae*, shows a high production of IAA (17.934 µg/mL) in culture medium with optimized tryptophan (0.2g L^{-1}); while our strain N2 also presents a high production of IAA (596.9 mg L^{-1}) in culture medium with standard tryptophan (10 g L^{-1}), as well as our strain N1g shows a production of 743.7 mg L^{-1}; therefore, under optimized conditions of temperature, pH, or another type of supplement, as in the study by Panigrahi et al. 2020, our strains could show a better production.

According to Panigrahi et al. 2020, strain OS03 can also grow under osmotic stress (40–45% PEG 6000) in the medium; while in our study, we also evaluated the production of IAA under osmotic stress in the medium equivalent to an osmotic potential of -2MPa, and the N1g and N2 strains, even under these severe stress conditions, showed a high production of 81.3 mg L^{-1} and 101.8 mg L^{-1} respectively.

Ahmed et al. 2021 studied the strain *Enterobacter* sp. / *Leclercia adecarboxylata* PAB19 a drought-tolerant rhizobacteria that can tolerate high level of drought (18% PEG-6000), and the IAA activity of the strain increases with the level of PEG up to a concentration of 15%, where a maximum of 176.2 µg mL^{-1} of IAA was recorded (a 23% increase over the non-PEG-treated control). While N2 and N1g from our study can tolerate a higher level of drought, and still produce considerable amounts of IAA for plant development.

Phosphorus (P) is an important plant nutrient and is involved in almost all aspects of plant metabolism, including synthesis of leaf pigments, respiration, and energy transfer (Zaidi et al. 2017). Many drought-tolerant rhizobacteria, including the *Bacillus* genus, solubilize soil P under water stress conditions (Kumar et al. 2016); and when they are inoculated in edible crops with water stress, their growth and development increase, such is the case of *Bacillus amyloliquefaciens* (Danish and Zafar-ul-Hye 2019).

In our study, we noted how the activity of P solubilization by strains N1g and N2 decreased under conditions of stress due to severe drought, showing only 5.73 mg L^{-1} (N1g) and 4.54 mg L^{-1} (N2), when under non-stress conditions 194.9 mg L^{-1} (N1g) and 468.5 mg L^{-1} (N2) are obtained. Therefore, we assume that under severe drought stress conditions, the ability of these strains to solubilize phosphorus is not precisely an element that decisively affects drought tolerance.

ACC deaminase-producing bacteria sequester and break down plant ACC to provide nitrogen and energy. In addition, by eliminating the ACC, the bacteria mitigate the negative effects of ethylene, relieving the stress of the plants and enhancing their development (Glick, 2012). Considering this, we evaluated the

ACC deaminase activity of the N1g and N2 strains in the presence of drought stress. Both strains, despite decreasing their activity, showed a good response for ACC deaminase at that osmotic potential equivalent to -2MPa.

Previous investigators reported ACC deaminase production by drought-tolerant rhizobacteria subjected to water deficit; and according to Ansari et al. 2021 the exogenous expression of ACC deaminase by *P. azotoformans* FAP5 improved the growth and biochemical attributes of wheat plants raised in soils with different levels of water stress. Danish et al. 2020 reported a similar inoculation impact where ACC deaminase-producing PGPR strains mitigated the adverse effects of drought stress and increased photosynthetic rate, stomatal conductance, and nutritional value of maize. Taking these results into consideration, the positive activity for ACC deaminase of our strains N1g and N2 under severe drought stress may enhance the characteristics of drought tolerance and plant growth promotion in a stress situation in plants.

Another positive feature in our results was ammonium production by the bacterial strains. Currently, nitrogenous fertilizers are applied in the form of ammonium through the process of biological nitrification, transforming ammonium into accessible nitrates for plant uptake (Lu et al. 2019). Therefore, the evaluation of ammonium production was interesting for our research. The two bacteria in our study were able to produce plant-assimilable ammonium, allowing nitrogen accumulation in plant tissues, restoration of photosynthetic efficiency, and cellular processes that help plants overcome stress conditions due to drought.

On the other hand, the availability of potassium in plants is essential for different processes such as photosynthesis, regulation of stomatal opening and closing, enzyme activation, protein synthesis, and other cellular processes that promote plant development and productivity (Ahmad and Zargar 2017). Potassium solubilizing bacteria allow the conversion of insoluble forms or mineral compounds of potassium into forms available to plants (Valle et al. 2022). In the present study it was shown that our N2 strain has the potential to solubilize potassium. This effect improves the mineral increase in plants, favoring plant nutrition and nutrient absorption processes in dry conditions.

Plants are more vulnerable to pathogens during abiotic stress, so a solution to provide cross-protection against phytopathogens in a stress situation is always appreciable. Our bacterial strains also showed positive activity for siderophores. Low molecular weight siderophores can bind most of the available iron in the rhizosphere with high avidity, which inhibits the proliferation of fungal pathogens in the roots of host plants due to lack of available iron (Saikia et al. 2018). A previous report looked at siderophores and their relationship to drought resistance, and found that the strain producing the highest level of siderophores is associated with excellent drought resistance of host plants (Ashry et al. 2022). Some siderophore-producing bacteria have been found in DXWR, with *Bacillus* being one of the main genera (Sultana et al. 2021; Zhang et al. 2021), which is in harmony with our findings in DXWR with strain N1g.

Hydrogen cyanide (HCN) production is another protective trait against pathogen entry into plants during stress conditions. HCN-producing bacteria induce systemic resistance in plants by acting as extracellular

signals, subsequently triggering a series of internal processes; and ultimately, the translocated signal is perceived by plant cells that activate cellular defense mechanisms (Saikia et al. 2018). The N2 strain of our study showed positive activity to produce HCN. Therefore, a bacterial formulation from the mixture of N1g and N2 strains would have additional advantages to protect plants against attacks by phytopathogens during abiotic stress.

The use of synergistically acting mixed microbial inoculants is beneficial for higher yields and rapid results; furthermore, the combination of bacterial cells has the potential to supply more nutrients to host plants, thus exerting greater plant growth promoting effects (Saikia et al. 2018).

According to (Ashry et al. 2022), the coinoculation of two or more strains leads to a greater tolerance to drought stress in plants. The N1g and N2 strains from our study are compatible with each other, and the results of the *in vivo* experiments revealed significant improvements in the growth promotion of the test plants under drought conditions, agreeing with Saikia et al. (2018), who through the combined action of *O. pseudogrignonense* RJ12, *Pseudomonas* sp. RJ15 and *B. subtilis* RJ46 alleviated water stress in black gram and garden pea plants. Inoculation of black gram and garden pea plants with the consortium resulted in increased root length elongation, increased total foliar chlorophyll synthesis, and accelerated production of antioxidant enzymes.

Mahreen et al. 2023 found that inoculation of a bacterial consortium (*Bacillus subtilis* NM-2, *Brucella haematophilum* NM-4, and *Bacillus cereus* NM-6) under drought-stressed conditions significantly improved rice seedling growth as compared to non-inoculated stressed plants. The inherent plant growth promoting traits of individual bacteria may provide an indirect mechanism to alleviate water stress in tested plants by providing sufficient phosphate, iron, available nitrogen, and cross-protection against pathogen entry. Therefore, the use of microbial consortia that can induce drought tolerance and also improve plant growth could be a sustainable strategy for agriculture.

In addition, one of the bacterial strains used in our mixture is a *Bacillus*, which, due to its ability to produce endospores, is a resistance structure, which constitutes a potentiality to be taken into account for formulations of a bioproduct. The *Bacillus* genus is interesting due to its ability to produce a large number of enzymes, phytohormones, and metabolites that can favor plant growth under biotic and abiotic stress conditions (De Lima et al. 2019). In addition, an increase in the generation of biofilms by *Bacillus* spp. under stress conditions is favored by the production of EPS under stress conditions (Ashry et al. 2022).

Enterobacter spp. also has been reported as a rice rhizobacteria; and Saengsanga (2017) studied *Enterobacter* sp. NRRU-N13, and its plant growth-promoting abilities such as IAA production and P solubilizing activity. This strain, after being inoculated in rice seedlings, it increased shoot lengths and dry weights. Niu et al. (2018) have reported *Enterobacter hormaechei* and *Enterobacter asburiae* as drought-tolerant strains from the rhizospheric soil of Foxtail millet (*Setaria italica* L.), these bacteria show capacities such as EPS production, ACC deaminase activity, and enhance plant growth under drought stress conditions.

Our study with the bacterial strains N1g and N2, agrees with the antecedents shown, and encourages us to continue studying the mechanism of action and the potential of these bacterial genera for drought tolerance.

According to Barnawal et al. 2019, EPS-producing strains detoxify free radicals, and reduce the negative effects of reactive oxygen species (ROS) in plants through the activation of antioxidant defense, reflected in the enzymatic activity (CAT, POD and SOD). Catalase induces the dismutation of H_2O_2 into water and molecular O_2 (Singh et al. 2020); and POD degrades H_2O_2 by oxidizing co substrates such as phenolics and antioxidants (Ilyas et al. 2020). The high content of MDA is an important indicator of the negative effects of ROS in plants, since it is responsible for cell membrane damage and the main cause of oxidative damage (Valle et al. 2022).

In the current study, we found that rice plants inoculated with the N1g + N2 bacterial formulation showed a higher accumulation of antioxidants (SOD, CAT and POD) under water stress compared to non-inoculated stressed plants (Fig. 4b-d). While the MDA content of the shoots was significantly reduced by 65.9% in plants inoculated with N1g + N2 compared to control plants not inoculated under drought conditions (Fig. 4a), corroborating the potential of these antioxidants to minimize oxidative damage and achieving plants more drought tolerant. This effect may be due to the presence of drought-tolerant strains that produce EPS, which reduce oxidative stress induced by water stress, improving water retention, and favoring the development of rice seedlings.

These results agree with Rezazadeh et al. 2019 and Gowtham et al. 2020, who also reported a significant increase in antioxidant enzymes by inoculating EPS-producing bacterial strains under drought stress conditions. Antioxidant activity is not only critical during acute drought stress, but also interferes with recovery from water limitation and dehydration resuscitation (Laxa et al. 2019). Similarly, two drought-tolerant PGPR strains, *Bacillus cereus* P2 and *Planomicrobium chinense* P1, reduced MDA content when inoculated into *Helianthus annus* under water stress conditions (Khan et al. 2018 a). Also, under water stress, decreases in MDA content were observed in the foliage of chickpea plants inoculated with three drought-tolerant PGPR strains, namely *B. subtilis*, *B. thuringiensis* and *B. megaterium* (Khan et al. 2018 b). These findings support the use of desiccation tolerant strains to improve drought tolerance of plants by changing antioxidant activity and stress metabolites (MDA) under water stress situations.

In general, the growth parameters of the plants in our study were severely reduced under drought stress compared to the well-irrigated control. But on the other hand, these parameters increased in plants inoculated with the N1g + N2 bacterial formulation, even under drought stress. These increases, even in the presence of water stress, were likely due to the production of IAA and other active plant growth promoting chemicals, which help plants by promoting symbiosis and root morphogenesis in the presence of drought stress. Siderophore, ACC deaminase, HCN and NH_4^+, for example, are involved in plant development (Asghari et al. 2020).

Therefore, we consider that, under drought stress conditions, these bacterial strains with EPS secretion capacity activate the antioxidant defense machinery of rice plants, colonize the rhizosphere, adhere to the root surface, and maintain moisture content. These strains have adhesive properties, and form stable aggregates that facilitate nutrient uptake and water availability, which in turn improves development and growth of rice plants.

Conclusions

Dongxiang wild rice's rhizosphere harbors drought-tolerant bacteria capable of promoting plant growth. As an organic strategy for the growth of cultivated rice, inoculation with a formulation based on two bacterial strains tolerant to drought *Bacillus* sp. N1g and *Enterobacter* sp. N2, capable of withstanding a high concentration of PEG-6000 while secreting substances that protect the cell membrane such as EPS, avoided drought stress in our experimental conditions by activating protective mechanisms in plants such as the production of antioxidant enzymes and the decrease in the lipid peroxidation. The N1g + N2 bacterial formulation offers a potentially attractive, agronomically viable alternative that deserves future optimization studies for rice cultivation under drought conditions.

Abbreviations

PGPR Plant growth promoting rhizobacteria

MDA Malondialdehyde

DXWR Dongxiang wild rice

EPS Exopolysaccharides

LB Luria-Bertani culture medium

TSB Trypticase Soy Broth

H_2SO_4 Sulfuric acid

IAA Indoleacetic acid

RH Relative humidity

PEG 6000 Polyethylene glycol 6000

NaCl Sodium chloride

$FeCl_3$ Iron trichloride

$HClO_4$ Perchloric Acid

P Phosphorus

NBRIP National Botanical Research Institute's phosphate growth medium

NH_4^+ Ammonium production

K potassium

DF Dworkin and Foster salts media

HCN Hydrogen cyanide

CK(-)WL drought + foliar application of sterile water

PB Biofertilizer P

EM Efficient Microorganisms

CK(+) Plants under normal water conditions

TBA thiobarbituric acid

FW Fresh weight

TCA Trichloroacetic acid

SOD Superoxide dismutase

CAT Catalase

POD Peroxidase

NBT Nitroblue tetrazolium solution

PGPM Plant growth promoting microorganism

Declarations

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Conflict of interest

The authors declare that there are no conflicts of interests.

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Figures

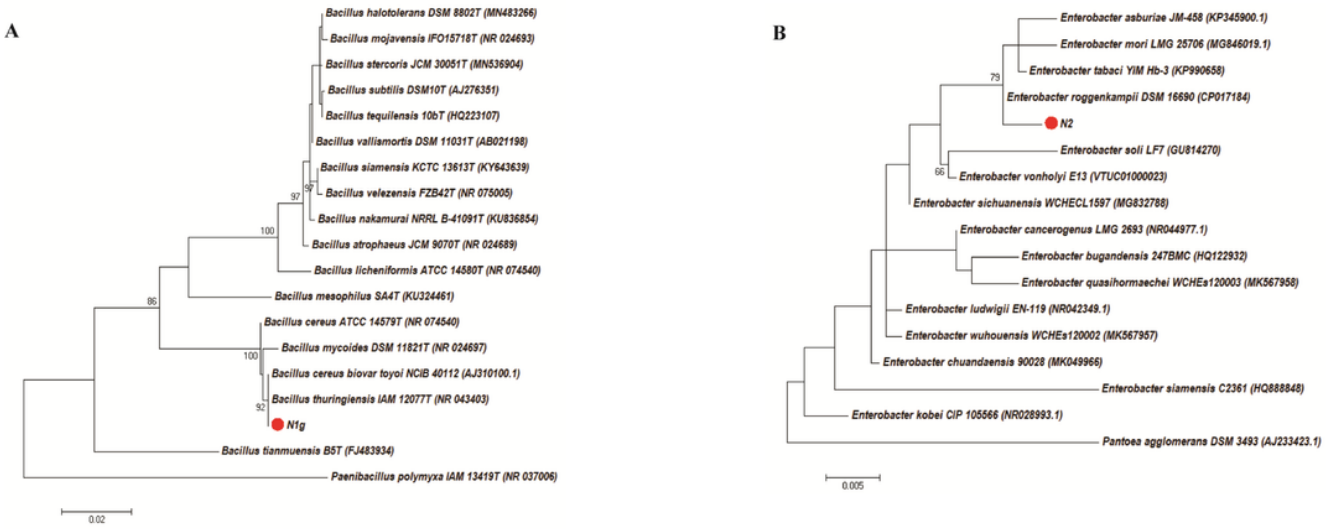

Figure 1

Maximum likelihood tree showing phylogenetic relationship between the bacterial taxa identified in this study and their representative species from NCBI database.Bootstrap values ≥70% (based on 1000 replications) were displayed at the node. **A,** N1g; **B**, N2. 16S sequences of *Paenibacillus polymyxa* IAM 13419T and *Pantoea agglomerans* DSM 3493 were used as outgroups for N1g and N2 respectively.

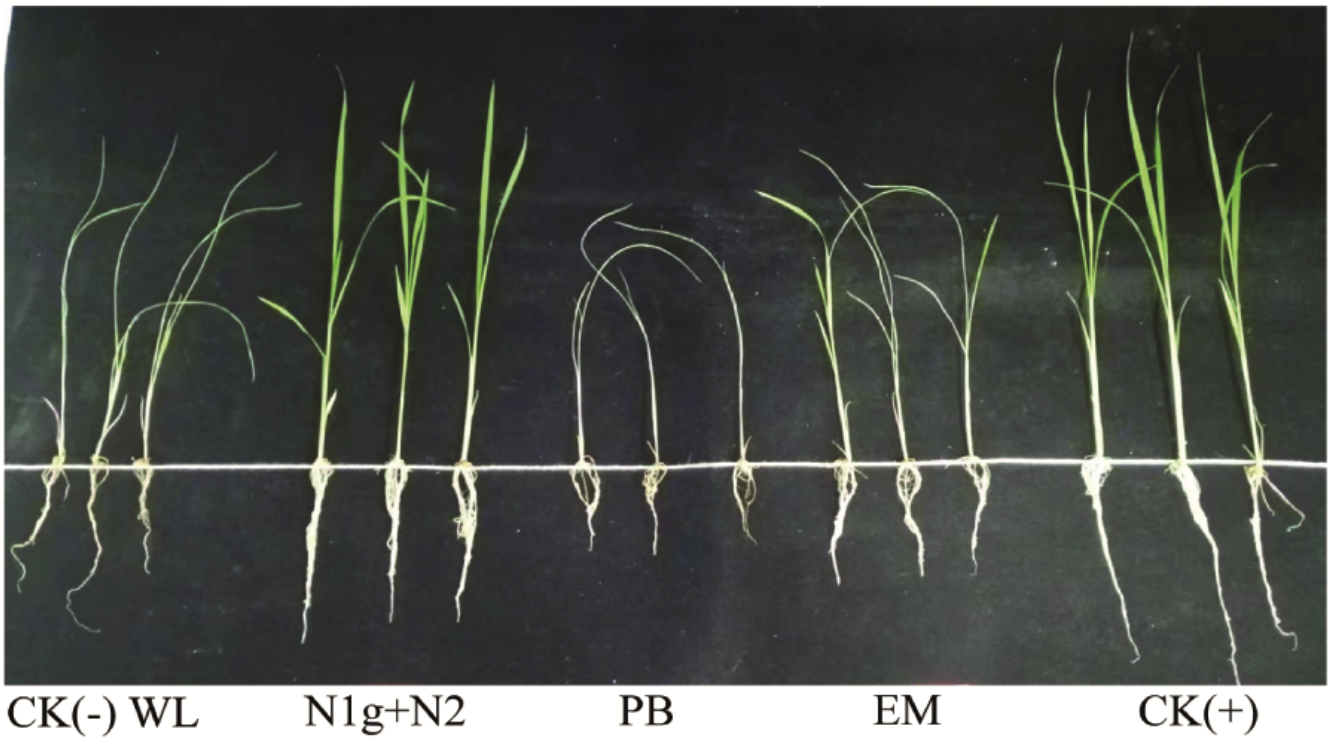

Figure 2

N1g+N2 bacterial formulation effect on rice seedlings exposed to drought stress (10 days). The treatments are CK(-)WL, drought + foliar application of sterile water; N1g+N2, drought + foliar application of the bacterial formulation; PB, drought + soil inoculation of the commercial product; EM, drought + soil inoculation of the commercial product; CK(+),plants under normal water conditions.

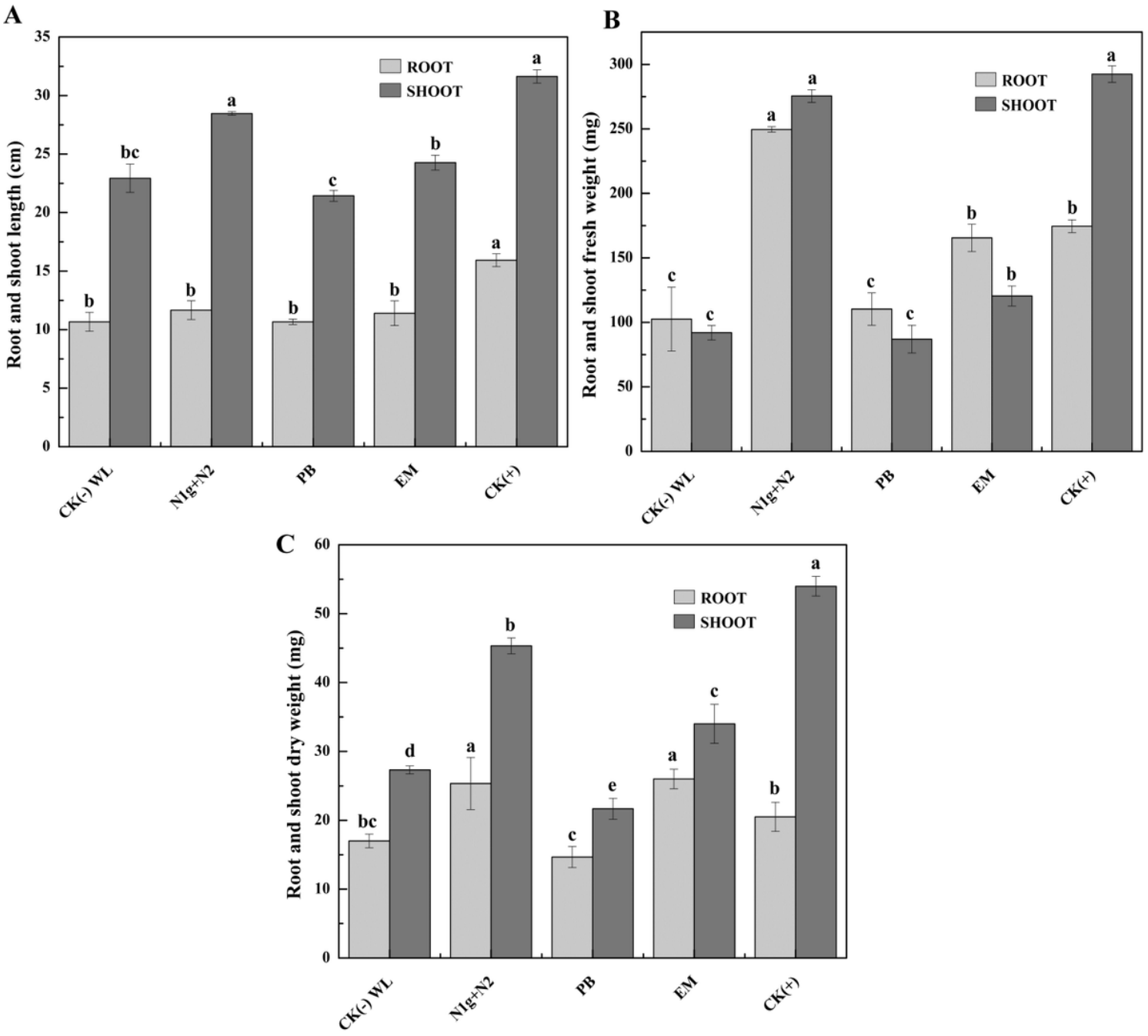

Figure 3

N1g+N2 bacterial formulation effect on morphological parameters of rice seedlings exposed to drought stress (10 days). **A,** Root and shoot length; **B,** Root and shoot fresh weight; **C,** Root and shoot dry weight. The treatments are CK(-)WL, drought + foliar application of sterile water; N1g+N2, drought + foliar application of the bacterial formulation; PB, drought + soil inoculation of the commercial product; EM, drought + soil inoculation of the commercial product; CK(+),plants under normal water conditions. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).

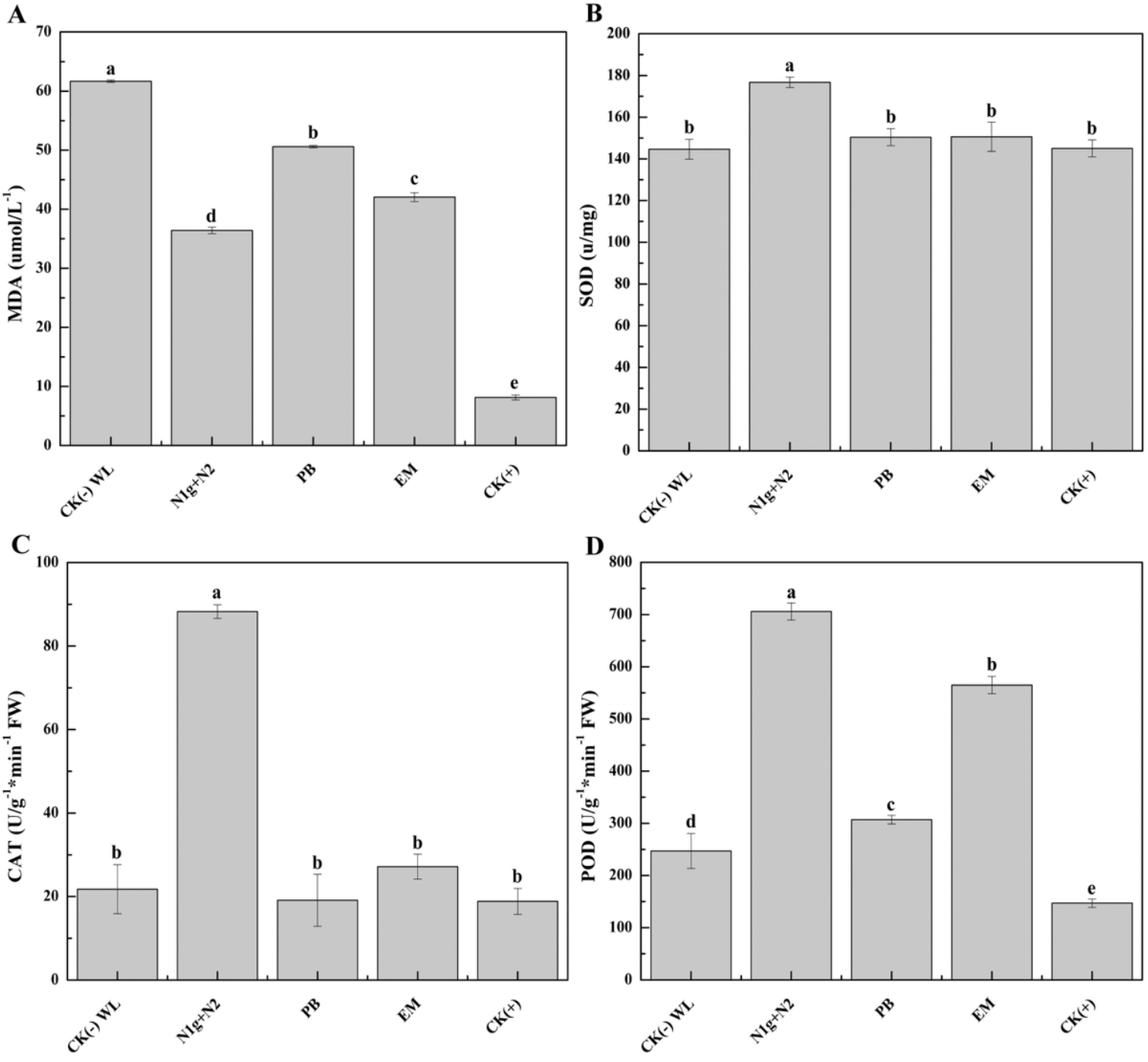

Figure 4

N1g+N2 bacterial formulation effect on antioxidant defense of rice seedlings exposed to drought stress (10 days). **A,** MDA content; and antioxidants enzymes **B,** SOD; **C,** CAT; **D,** POD. The treatments are CK(-)WL, drought + foliar application of sterile water; N1g+N2, drought + foliar application of the bacterial formulation; PB, drought + soil inoculation of the commercial product; EM, drought + soil inoculation of the commercial product; CK(+),plants under normal water conditions. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).