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The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa L.*)

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ABSTRACT

The potential of seed endophytic microbes to enhance plant growth and resilience is well recognized, yet their role in alleviating cold stress in rice remains underexplored due to the complexity of these microbial communities. In this study, we investigated the diversity of seed endophytic microbes in two rice varieties, the cold-sensitive CB9 and the cold-tolerant JG117. Our results revealed significant differences in the abundance of Microbacteriaceae, with JG117 exhibiting a higher abundance under both cold stress and room temperature conditions compared to CB9. Further analysis led to the identification of a specific cold-tolerant microbe, *Microbacterium testaceum* M15, in JG117 seeds. M15-inoculated CB9 plants showed enhanced growth and cold tolerance, with a germination rate increase from 40 % to 56.67 % at 14℃ and a survival rate under cold stress (4℃) doubling from 22.67 % to 66.67 %. Additionally, M15 significantly boosted chlorophyll content by over 30 %, increased total protein by 16.31 %, reduced malondialdehyde (MDA) levels by 37.76 %, and increased catalase activity by 26.15 %. Overall, our study highlights the potential of beneficial endophytic microbes like *M. testaceum* M15 in improving cold tolerance in rice, which could have implications for sustainable agricultural practices and increased crop productivity in cold-prone regions.

1. Introduction

Abiotic stresses such as cold, heat, drought, and salinity threaten global agricultural productivity, causing about 70 % yield losses of major crops (Zubair et al., 2019). With global climate change, enhancing crop adaptability to extreme weather is crucial. Frequent extreme climate events, such as cold spells and frosts, particularly affect temperate and high-altitude regions, posing a direct threat to global food security (Zhang et al., 2018). Rice (*Oryza sativa L.*), a staple food for nearly half of the global population. Although rice is primarily grown in tropical and subtropical regions, it is also extensively cultivated in some temperate areas, such as Northeast China. In these regions, cold spells or climate variability can induce cold stress during early growth stages, significantly impacting yields. Cold stress interferes with the plant's metabolic processes, damages chloroplasts, reduces photosynthetic activity, and hampers the synthesis of proteins, lipids, and carbohydrates (Ma et al., 2015; Hsu and Hsu, 2019; Manasa et al., 2022). Cold stress affects rice throughout its life cycle, causing delayed germination, reduced seedling vigor, pollen sterility, and decreased grain quality (Guo et al., 2020; Li et al., 2022; Usman et al., 2023). Seed germination and vigorous seedling growth are crucial for stable crop development, because robust seedling growth under cold stress leads to improved crop performance and resilience (Zhang et al., 2018; Zhao et al., 2020). Enhancing cold tolerance in rice not only helps stabilize production and ensure global food security but also enables cultivation in higher altitudes and latitudes, thus expanding the geographic range of rice cultivation and contributing to greater agricultural resilience.

To combat cold stress in rice, researchers have investigated

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traditional breeding, genetic engineering, physical and chemical treatments, and optimized cultivation practices (Zeng et al., 2019; Xu et al., 2020). However, these strategies have limited effectiveness, are costly, and have potential environmental repercussions. A more promising approach is the use of plant growth-promoting bacteria (PGPB), which promote plant growth and stress tolerance via mechanisms such as nitrogen fixation, solubilization of phosphate, regulation of hormones, and strengthening of defense responses (Vega-Celedón et al., 2021). Recent research trends highlight the significance of biopriming using microbial inoculants for sustainable agriculture, demonstrating their potential in enhancing crop resilience and productivity(Thakur et al., 2023; Thakur and Yadav, 2023). Applying cold-tolerant PGPB as inoculants, especially in temperate and high-altitude regions, has marked potential (Bisht et al., 2013; Puranik et al., 2022; Niu et al., 2023). Cold-tolerant PGPB contribute to the resilience of agricultural systems, which is essential for ensuring food security in regions vulnerable to climate change (Mishra et al., 2012; Wu et al., 2019; Li et al., 2021). Cold-tolerant microbial inoculants have been reported to be effective in cold stress scenarios; for example, *Burkholderia phytofirmans* PsJN was found to improve the cold tolerance of grapevine and *Arabidopsis*, and cold-tolerant *Pseudomonas* and *Acinetobacter* strains were reported to mitigate cold stress in wheat (Ait Barka et al., 2006; Su et al., 2015; Vega-Celedón et al., 2021).

There is a lack of research on the use of microbes to mitigate cold stress in rice. Most studies have focused on microbes from cold environments, neglecting seed endophytes (Mishra et al., 2012; Yadav et al., 2015). Seed endophytes are crucial throughout the plant life cycle, assembling the plant microbiome; persisting in leaves, stems, and roots; and influencing plant health and productivity (Berg and Raaijmakers, 2018; Kim and Lee, 2021; Shao et al., 2021; War et al., 2023). The genotype of the host significantly affects microbiome assembly; the host plant shapes the seed microbiome structure and regulates stress-related traits (Rybakova et al., 2017). Thus, rice seeds of different varieties can be used as sources of low-temperature-adapted endophytes.

Microbacteriaceae, a diverse Gram-positive bacterial family of the class Actinobacteria, are common plant endophytes (Evtushenko, 2015; Vasilenko et al., 2018; Tarlachkov Sergey et al., 2020). Some members have plant growth-promoting, bioremediation, and biocontrol properties (Evtushenko and Takeuchi, 2006). For instance, *Microbacterium testaceum*, originally isolated from rice, enhances plant growth and has potential for biocontrol (Takeuchi and Hatano, 1998; Zinniel Denise et al., 2002; Zinniel et al., 2008). However, its role in enhancing plant cold tolerance is unclear. Plant growth-promoting bacteria like Microbacteriaceae play a critical role in nutrient management, which is essential for plant survival and growth, particularly under stress conditions. Among the key nutrients, phosphorus (P) is vital for various plant developmental processes. However, most soil phosphorus is in insoluble forms unavailable to plants, making microbial phosphorus solubilization crucial (Khan et al., 2010; Kour et al., 2021; Zhang et al., 2021; Zhao et al., 2022). Phosphate-solubilizing bacteria (PSB) increase phosphorus availability, promoting growth and stress tolerance (Rizvi et al., 2021). PSB are a sustainable and economical substitute for chemical fertilizers, especially in cold regions where nutrient cycling is slow (Zaidi et al., 2014). They can promote sustainable agriculture, improve soil health, and reduce the environmental footprint of agriculture (Vyas et al., 2010; Kour and Yadav, 2023b; Kour and Yadav, 2023a). However, the mechanisms by which cold-tolerant PSB promote rice growth and cold tolerance are unclear (Dasila et al., 2023; Kour and Yadav, 2023b).

While there has been extensive research on cold-tolerant microbes from extreme environments, there is a significant gap in understanding the role of seed endophytes in cold tolerance, especially in rice. Current studies often overlook the unique potential of these microbes, which are naturally adapted to the plant host environment and could be crucial throughout the plant's life cycle. This study aims to explore seed endophytic microbes, particularly their role in enhancing cold tolerance in rice. By isolating and characterizing Microbacterium testaceum M15 from rice seeds, we aim to fill this research gap and utilize native endophytes to improve rice cold tolerance. This approach leverages naturally adapted microbes and cross-variety inoculation to exploit different genetic backgrounds, thereby reconstructing the host microbiome and enhancing plant resilience to cold stress. To explore the role of seed endophytic microbes in enhancing rice cold tolerance, understand the mechanisms by which these microbes promote plant growth under cold stress, and develop microbial inoculants that can improve rice resilience across different environmental conditions, we conducted a comprehensive study focusing on these aspects. This study aims to provide insights into the potential applications of such inoculants in improving crop performance and adaptability, contributing to sustainable agricultural practices.

2. Materials and methods

2.1. Evaluation of cold tolerance

2.1.1. Plant materials and growth conditions

We used two *Japonica* rice varieties, cold sensitive rice variety (CB9) and cold tolerant rice variety (JG117), sourced from the Rice Research Institute of Jilin Academy of Agricultural Sciences, China. The seeds were subjected to surface sterilization by immersion in 75 % ethanol for 10 minutes, followed by rinsing with sterile water. They were then treated with 10 % sodium hypochlorite for another 10 minutes to ensure thorough disinfection, followed by multiple rinses with sterile water to remove any remaining residues. The sterilized seeds with husks were soaked in sterile water at 30°C for 3 days and allowed to germinate on moistened filter paper in Petri dishes for 1 day. The germinated seeds were subsequently planted in glass tubes containing half-strength Murashige and Skoog (MS) nutrient solution and 0.4 % agar. The growth conditions were 26°C with a 14 h light and 10 h dark cycle for 1 week. Then the plants were exposed to cold stress at 4°C for 1 week, followed by recovery at 26°C for 1 week. The experiment was conducted with five replicates for each tested condition and variety. The survival rate, root length, shoot length, and fresh weight were measured. The survival rate was calculated using the formula: survival rate (%) = (number of surviving seedlings ÷ total number of seedlings) × 100 %.

2.2. Endophytic microbial diversity in rice seeds and seedlings

2.2.1. Sample preparation for detection

Two grams of surface-sterilized rice seeds from the varieties JG117 and CB9 were ground into fine powders using a sterile mortar and pestle. Each experimental group consisted of five replicates.

For seedling cultivation, separate sets of seeds from both varieties were grown in sterile half-strength MS nutrient solution containing 0.4 % agar under a 14-hour light/10-hour dark cycle at 26°C. After 7 days of growth, seedlings were exposed to cold stress at 4°C for an additional 7 days under the same light conditions. We maintained five replicates per variety, with five seedlings per tube, ensuring the experiment was conducted entirely under axenic conditions. The seedlings were surface-sterilized and homogenized using a sterile mortar and pestle.

Genomic DNA was extracted from the homogenates of both rice seeds and seedlings to assess microbial diversity. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806 R (5'-GGACTACNNGGGTATCTAAT-3'). Sequencing was conducted on the Illumina MiSeq platform employing a PE250 strategy (Illumina, Inc.) at Beijing Allwegene Technology Co., Ltd. The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1069338.

2.3. Microbiological characteristics of Microbacteriaceae

2.3.1. Isolation and screening of bacterial strains

Two grams of JG117 rice seeds were surface sterilized as described previously and ground into fine powder. This powder was suspended in 5 mL sterile 0.9 % saline and thoroughly mixed to resuspend endophytic microorganisms. One milliliter of the seed suspension was transferred to a sterile tube containing 9 mL sterile phosphate-buffered saline (PBS) to prepare a 1:10 dilution. Next, serial dilutions were performed to create 1:100, 1:1000, and 1:10,000 dilutions. From each dilution, 100 μL was spread onto Reasoner's 2 A (R_2A) nutrient agar using a sterile spreader. The plates were incubated at 10°C for 1 month to facilitate the growth of slow-growing endophytic bacteria. Colonies with varied morphology were repeatedly streaked onto fresh Luria-Bertani (LB) agar to isolate pure cultures. Bacterial isolates were preserved in LB medium containing 20 % glycerol at −20℃.

2.3.2. Identification of isolated strains

The 16S rRNA genes of the bacterial isolates were amplified using the primers 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were sequenced by Tsingke Biological Technology Co., Ltd. The resulting sequences were compared to those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) for the identification and screening of Microbacteriaceae.

2.3.3. Analysis of bacterial cold tolerance

Bacterial strains were cultured overnight at 37°C in 3 mL LB medium with shaking at 200 rpm. A 1 % aliquot of this culture was transferred to fresh 50 mL LB medium and incubated at 4°C with shaking at 200 rpm for 13 days. Daily growth was monitored by measuring the optical density at 600 nm (OD_{600}), with three replicates per bacterium. OD_{600} values were plotted to assess the cold tolerance of the bacterial strains.

2.3.4. Determination of IAA production by strains

The production of indole-3-acetic acid (IAA) by bacterial strains was assessed using a modified qualitative method described previously (Gordon and Weber, 1951). Bacterial isolates were grown overnight in 3 mL liquid LB medium. Following this, 1 % of the overnight culture was transferred to 3 mL of LB medium supplemented with 0.5 mg/mL L-tryptophan and incubated at 37°C for 24 hours. To evaluate IAA production under low-temperature conditions, a 30 % inoculum from the same overnight culture was transferred into 3 mL of LB medium with 0.5 mg/mL L-tryptophan and incubated at 4°C for 7 days.

After incubation, the cultures were centrifuged at 12,000 g for 10 minutes to collect the supernatant. A mixture of 0.5 mL supernatant and 0.5 mL Salkowski reagent was incubated in the dark at 25°C for 30 min. The IAA concentration was determined by measuring the absorbance at 530 nm using the SpectraMax M2 spectrophotometer (Molecular Devices), with three replicates per bacterium. The IAA level was quantified using a standard curve generated with known IAA concentrations (Bric et al., 1991)

2.3.5. Analysis of phosphate solubilization

The ability of bacterial strains to solubilize phosphate was evaluated using a modification of the method of Nautiyal (1999). Individual bacterial colonies were cultured overnight in 3 mL LB medium at 37°C with shaking at 200 rpm. A 1 % aliquot of this culture was transferred to 3 mL National Botanical Research Institute phosphate (NBRIP) growth medium and incubated at 37°C with shaking at 200 rpm for 24 h. To assess phosphate solubilization under low-temperature conditions, a separate experiment was conducted where a 1 % aliquot of the same culture was transferred to 3 mL of NBRIP medium and incubated at 4°C with shaking at 200 rpm for 7 days. The experiment included three replicates per bacterium. The concentration of soluble phosphate in the supernatant was measured by anti-colorimetry (Murphy and Riley, 1962) with reference to a standard curve prepared using known concentrations of K_2HPO_4.

2.3.6. Determination of siderophore production

The production of siderophores by bacterial strains was assessed using a modification of a previous reported method (Schwyn and Neilands, 1987). Individual bacterial colonies were cultured overnight in 3 mL of LB medium at 37°C with shaking at 200 rpm. After incubation, a 1 % aliquot of this culture was transferred to 3 mL of Chrome Azurol S (CAS) basal growth medium and incubated again at 37°C with shaking at 200 rpm for 24 hours. To evaluate siderophore production under low-temperature conditions, a separate experiment was conducted. A 30 % aliquot of the same overnight culture was transferred to 3 mL of CAS basal growth medium and incubated at 4°C with shaking at 200 rpm for 7 days. The supernatant of the bacterial culture was mixed with an equal volume of Chrome Azurol S (CAS) reagent and allowed to react at room temperature for 1 h. The experiment included three replicates per bacterium. The OD_{630} of the mixture (As) was measured, and the OD_{630} of uninoculated medium mixed with CAS reagent served as the reference value (Ar). The relative siderophore content was calculated using the formula: siderophore units (%) = (Ar – As) ÷ Ar × 100 (Machuca and Milagres, 2003).

2.4. Bacterial inoculation on rice seedlings for growth promotion and cold tolerance

2.4.1. Preparation of bacterial suspensions

Single bacterial colonies were inoculated into 50 mL LB broth and incubated overnight at 37℃ with shaking at 200 rpm. The culture was centrifuged at 5000 g for 10 min, and the bacterial pellet was resuspended in sterile 0.9 % saline to a final concentration of approximately 10^8 CFU/mL.

2.4.2. Rice seed treatment

Surface-sterilized CB9 rice seeds were soaked in prepared bacterial suspension at 30°C with shaking at 120 rpm for 6 h. Control seeds were treated with sterile 0.9 % saline. The treated seeds were placed on moist filter paper in sterile Petri dishes and incubated in the dark at 30°C for 1 day to germinate.

2.4.3. Growth promotion and colonization assay

Germinated seeds were transferred to pots containing sterile vermiculite and maintained at 26°C under a 14 h light/10 h dark photoperiod for 1 month. Each seedling was watered with 10 mL bacterial suspension or sterile water (control). For each treatment, three 9 cm square plant pots were prepared with nine seeds planted in each. The root length, shoot length, and fresh weight were recorded. For colonization assays, seedlings were cultivated in glass tubes containing half-strength sterile MS medium with 0.4 % agar at 26°C under the same photoperiod for 7 days. The experiment was conducted with three replicates for each tested condition. Samples were collected to evaluate endophytic microbial diversity as described previously.

2.4.4. Cold stress and analysis of survival rate

Seedlings at the three-leaf stage, treated with bacterial suspension or saline, were subjected to cold stress at 4℃ under a 14 h light/10 h dark photoperiod for 5 days. Subsequently, the seedlings were transferred to 26℃ to recover for 7 days. The experiment included three replicates per treatment. Survival rates were calculated as follows: survival rate (%) = (number of surviving seedlings ÷ total number of seedlings) × 100 %.

2.4.5. Low-temperature seed germination rate

Rice seeds treated with bacterial suspension and control seeds were placed in the dark at 14°C for 7 days. The experiment included three replicates per treatment. The germination rate was calculated using the formula: germination rate (%) = (number of germinated seeds ÷ total

number of seeds) × 100 %.

2.4.6. Analysis of chlorophyll content

Fresh rice leaves (0.1 g) were collected after exposure to cold stress as described previously. Each treatment included three replicates. Leaves were homogenized in an ice bath with 2 mL extraction buffer (ethanol: acetone = 4.5:4.5:1), centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. Chlorophyll content was measured at 663 and 645 nm using a spectrophotometer (Arnon, 1949). The concentrations of chlorophyll a, b, and total chlorophyll were calculated using the following formulae: chlorophyll a = (12.72 OD_{663} − 2.59 OD_{645}) × V/1000 W; chlorophyll *b* = (22.88 OD_{645} − 4.67 OD_{663}) × V/1000 W; total chlorophyll = (8.05 OD_{663} + 20.29 OD645) × V/1000 W, where V is the volume of the extraction liquid (mL) and W is the fresh weight of the leaves (g).

2.4.7. Analysis of MDA content

Fresh rice leaves (0.5 g) were collected after exposure to cold stress as described previously. Each treatment included three replicates. The leaves were homogenized in 5 mL 5 % trichloroacetic acid (TCA) in an ice bath, centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. Two milliliters of the supernatant were mixed with 2 mL 0.5 % thiobarbituric acid (TBA) in 0.1 % TCA, heated at 95°C for 30 min, and rapidly cooled. Absorbance was measured at 532, 600, and 450 nm. Malondialdehyde (MDA) content was calculated using the formula: MDA (μmol/g FW) = 6.45 (OD_{532} − OD_{600}) − 0.56 OD_{450} (Heath and Packer, 2022).

2.4.8. Analysis of total protein content

Fresh rice leaves (0.1 g) were collected after cold stress as previously described. Each treatment had three replicates. Leaves were homogenized in buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5 % Triton X-100, protease inhibitors), centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. The supernatant was mixed with Laemmli buffer (250 mmol/L Tris-HCl, pH 6.8, 8 % SDS, 4 % glycerol, 4 % β-mercaptoethanol, 0.01 % bromophenol blue), heated at 95°C for 5 min, and the protein content was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Solarbio, China) (Bradford, 1976).

2.4.9. Analysis of catalase (CAT) activity

Fresh rice leaves (0.1 g) were collected from each treatment group following exposure to cold stress. Each treatment included three replicates. Leaves were homogenized in an ice bath using a mortar and pestle with 1 mL phosphate buffer (50 mmol/L, pH 7.0). The homogenate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. A 0.1 mL aliquot of the supernatant was mixed with 0.9 mL 0.2 mol/L H_2O_2. The decrease in absorbance at 405 nm over 5 min was measured using a spectrophotometer. Phosphate buffer served as the control. Relative CAT activity was determined as described previously (Aebi, 1984).

2.4.10. Assessment of Exopolysaccharides (EPS) Production by M15

M. testaceum M15 was cultured in De Man, Rogosa, and Sharpe (MRS) broth, which contains beef extract, yeast extract, and peptone. Cultures were incubated at 37°C (normal) and 4°C(cold stress) in a shaking incubator at 220 rpm for 7 days. After incubation, cultures were centrifuged at 10,000 x g for 15 minutes at 4°C to separate cells from the supernatant. EPS was extracted using cold ethanol precipitation (Wingender et al., 1999; Ruas-Madiedo et al., 2002): three volumes of cold ethanol (95 %) were added to the supernatant, mixed, and incubated at 4°C overnight. The precipitate was collected by centrifugation at 10,000 x g for 20 minutes at 4°C, washed with cold ethanol, and dissolved in deionized water. MRS broth without bacteria served as a blank control. EPS was quantified by the phenol-sulfuric acid method (Dubois et al., 1956) at 490 nm, using a glucose standard curve. Results were expressed in mg of EPS per liter of culture. Experiments were performed in triplicate for reproducibility.

2.5. Bacterial genome and transcriptome analysis

2.5.1. Bacterial genome sequencing

Single bacterial colonies were cultured in 50 mL LB broth at 37°C with shaking at 200 rpm overnight. Next, the cultures were centrifuged at 5000 g for 10 min at 4°C, the supernatant was discarded, and the bacterial pellet was resuspended to extract genomic DNA. Genome library construction and sequencing were performed by Allwegene Technology Co., Ltd. (Beijing, China) using the Nanopore third-generation and Illumina second-generation sequencing platforms, with a sequencing depth ≥ 100×. Functional annotation of the genome was conducted using Blast2GO (Spain) for Gene Ontology (GO) analysis, EggNOG-mapper (Spain) for Clusters of Orthologous Groups (COG) analysis, and the KEGG Automatic Annotation Server (Japan) for KEGG pathway analysis. The complete genome sequence of *M. testaceum* M15 has been deposited in the NCBI GenBank database under the accession number CP143563. Detailed information about this project can be accessed using BioProject number PRJNA1069338 and BioSample number SAMN39615697.

2.5.2. Bacterial transcriptome sequencing

Single bacterial colonies were grown overnight in 3 mL LB broth at 37°C with shaking at 200 rpm, diluted 1 % into 50 mL LB broth, and incubated at 37°C or 4°C until an OD_{600} of approximately 1.0. For each condition, three independent replicates were used to ensure reproducible and accurate results. Transcriptome sequencing was performed on the Illumina Hiseq 4000 platform (Illumina) with the PE 150 strategy by Allwegene Technology Co., Ltd. (Beijing, China). The transcriptome data have been deposited in the NCBI SRA under accession number PRJNA1069338.

2.6. Phosphate solubilization and phosphorus supplementation

2.6.1. Measurement of pH change in NBRIP medium

Single colonies of M15 were cultured in 3 mL LB broth at 37°C with shaking at 200 rpm overnight. A 1 % inoculum was transferred to 50 mL NBRIP medium, with uninoculated NBRIP medium as the control. Each treatment was performed in triplicate. The cultures were incubated at 37°C with shaking at 200 rpm for 24 h, followed by centrifugation at 12,000 g for 10 min. The pH of the supernatant was measured using a digital pH meter.

2.6.2. Measurement of gluconic acid production

Single colonies of M15 were inoculated into 3 mL LB broth and incubated overnight at 37°C with shaking at 200 rpm. A 1 % inoculum was transferred to 200 mL NBRIP medium, with uninoculated NBRIP medium as the control. Cultures were incubated at 37°C with shaking at 200 rpm for 24 h, followed by centrifugation at 12,000 g for 10 min. The supernatant (500 μL) was combined with 500 μL methanol and filtered through a 0.22 μm membrane. A standard gluconic acid solution was prepared by dissolving 100 mg gluconic acid in 1 mL ultrapure water, followed by serial dilution to obtain a 1 mg/L standard solution. Gluconic acid was analyzed using liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ). LC-QQQ was conducted using an Agilent 1290 Infinity II LC system and an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies). A Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm, Thermo Fisher Scientific) was used. The mobile phases consisted of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, with gradient elution at a flow rate of 0.2 mL/min and an injection volume of 10 μL. The mass spectrometry conditions were a negative ion mode electrospray ionization (ESI) source with a capillary voltage of 3500 V, a drying gas flow rate of 10 L/min, a drying gas temperature of 250°C, and a nebulizer pressure of 35 psi. Detection was carried out in multiple reaction monitoring (MRM) mode for the ion pair

m/z 194.9 → 75.0, with the following parameters: precursor ion (Da): 194.9; product ion (Da): 75.0; dwell time, 12 ms; fragmentor voltage (V), 80; collision energy (V), 7; and polarity, negative (Wan et al., 2019; Zhang et al., 2024).

2.6.3. Phosphorus supplementation in rice

Surface-sterilized CB9 rice seeds were sown in sterile vermiculite pots and maintained in a controlled growth chamber set at 26°C with a 14 h light/10 h dark cycle. Phosphate solutions were prepared by dissolving pentoxide (P_2O_5) in deionized water to 0, 0.16, 0.32, 0.48, and 0.64 g/L. Rice seedlings were irrigated with these solutions, exposed to cold stress at 4°C for 5 days, and allowed to recover at 26°C for 7 days. The survival rates were recorded and calculated as: survival rate (%) = (number of surviving seedlings ÷ total number of seedlings) × 100 %. Each treatment included three replicates, with each replicate consisting of seedlings from different individual plants grown under the same conditions.

2.6.4. Analysis of leaf soluble phosphorus content

Surface-sterilized CB9 rice seeds were treated with bacterial suspensions as previously described (seed soaking and root irrigation). The seedlings were cultivated in a growth chamber at 26°C with a 14 h light/10 h dark cycle until the three-leaf stage. Each treatment was performed in triplicate. Approximately 0.5 g of seedling leaves were ground into fine powder using liquid nitrogen, mixed with 10 mL sterile deionized water, homogenized, and centrifuged at 12,000 g for 10 min at 4°C. The soluble phosphorus content in the supernatant was measured using the molybdenum blue method and quantified using a standard curve created with known concentrations of K_2HPO_4 (Murphy and Riley, 1962).

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 software (IBM Corp., Armonk, NY, USA). Data are expressed as means ± standard errors (SEs). The normality of the data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was conducted. When the assumption of homogeneity of variances was met, pairwise comparisons were performed using the least significant difference (LSD) test and Duncan's multiple-range test. When the assumption of homogeneity of variances was not met, Tamhane's T2 test was used. Statistical significance was set at $p < 0.05$ for all analyses. For comparisons between two groups, independent-sample *t*-tests were employed. The results were checked to ensure their robustness and reliability.

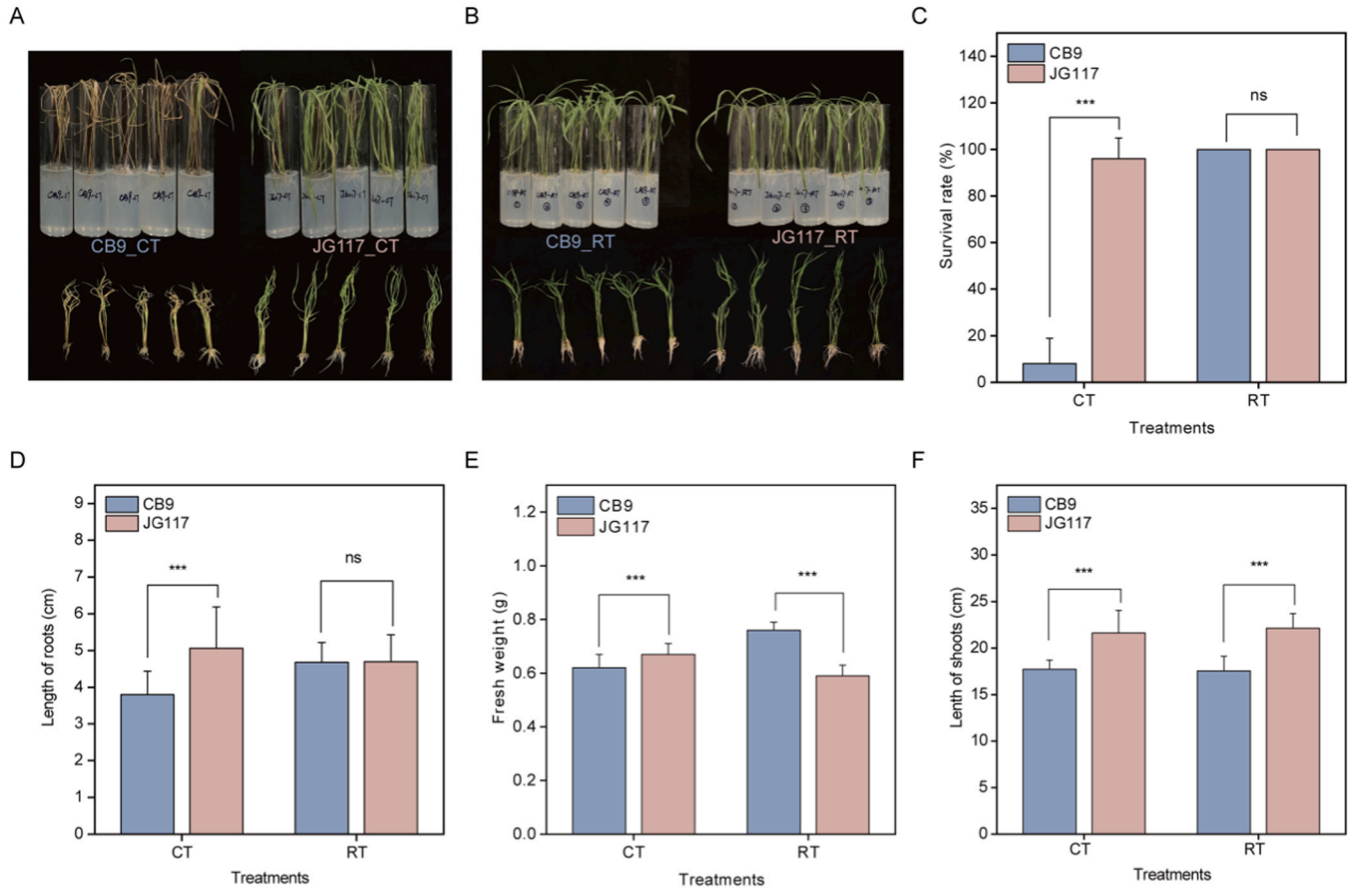

Fig. 1. Cold tolerance in the rice varieties JG117 and CB9 at the seedling stage. (A) Representative images of JG117 and CB9 seedlings after cultivation at normal temperature (26℃), cold stress (4℃), and recovery at normal temperature (26℃), each for 7 days. (B) Representative images of JG117 and CB9 seedlings grown under normal temperature conditions (26℃, RT) for 7 days. (C) Survival rates of JG117 and CB9 seedlings after cold stress treatment (CT) and normal temperature treatment (RT). (D) Root lengths of JG117 and CB9 seedlings after CT and RT. (E) Shoot lengths of JG117 and CB9 seedlings after CT and RT. (F) Fresh weights of JG117 and CB9 seedlings after CT and RT. CB9_CT: cold-treated CB9 seedlings, JG117_CT: cold-treated JG117 seedlings, CB9_RT: room temperature-treated CB9 seedlings, JG117_RT: room temperature-treated JG117 seedlings. Bar values are from five independent biological replicates. Mean values labeled with the same letter were not significantly different at $p < 0.05$.

3. Results

3.1. Cold tolerance of rice varieties

Initially, we evaluated the cold tolerance of two rice varieties, JG117 and CB9, during their seedling stages. After subjecting the seedlings to a sequence of 7 days at 26°C, followed by 7 days at 4°C, and a 7-day recovery at 26°C, JG117_CT (cold-treated JG117 seedlings) maintained green leaves and continued to grow normally. By contrast, CB9_CT (cold-treated CB9 seedlings) wilted and died (Fig. 1A). The survival rate of JG117 was significantly higher at 96 ± 8.94 % compared to CB9 at 8 ± 10.95 % (Fig. 1C). JG117 also had longer shoots and roots (21.64 ± 2.43 and 5.06 ± 1.13 cm) than CB9 (17.73 ± 1.00 and 3.80 ± 0.64 cm) (Figs. 1D, 1E). Additionally, JG117 had a higher fresh weight (0.67 ± 0.04 g) than CB9 (0.62 ± 0.05 g) (Fig. 1F). These results indicated that JG117 possessed superior cold tolerance to the cold-sensitive variety CB9.

Under room temperature conditions (26°C) for 7 days, both varieties exhibited normal growth and a 100 % survival rate (CB9_RT: room temperature-treated CB9 seedlings, JG117_RT: room temperature-treated JG117 seedlings) (Figs. 1B, 1C). The root lengths of JG117 and CB9 were similar (4.70 ± 0.73 vs. 4.68 ± 0.54 cm) (Fig. 1D), but JG117 had significantly longer shoots (22.14 ± 1.57 cm) compared to CB9 (17.55 ± 0.90 cm) (Fig. 1E). However, the fresh weight of JG117 (0.59 ± 0.04 g) was lower than CB9 (0.76 ± 0.03 g) (Fig. 1F), which may be attributable to the slender and elongated shoot structure of JG117, in contrast to the thicker and shorter shoots of CB9, resulting in different biomass accumulation.

3.2. Microbial diversity in rice seeds and seedlings

To evaluate the role of endophytic bacteria in enhancing cold tolerance in rice, we examined the microbial diversity of the seeds of two rice varieties, CB9 (cold sensitive) and JG117 (cold tolerant). The alpha diversity indices revealed distinct differences in species richness and community diversity between the two rice varieties. Specifically, the Chao1 index for CB9 averaged 236.29 ± 16.61, indicating higher microbial diversity than JG117 (average Chao1 index 156.30 ± 30.91) (Fig. 2A). Structural analysis by non-metric multidimensional scaling (NMDS) indicated a clear separation in microbial community structures between JG117 and CB9, indicating significant variation between the varieties (Fig. 2B).

Next, we examined the endophytic microbial diversity of seedlings of JG117 and CB9 cultured under different temperature conditions. JG117 seedlings at room temperature (JG117_RT) had higher microbial diversity (Chao1 index: 134.97 ± 67.75) compared to CB9 (CB9_RT) (57.14 ± 37.45) (Fig. 2C). Under cold stress, microbial diversity increased for both varieties, with CB9 (CB9_CT) (660.79 ± 34.07) being slightly higher than JG117 (JG117_CT) (610.80 ± 47.91) (Fig. 2C). NMDS analysis of beta diversity showed that temperature treatment (RT vs. CT) significantly affected the microbial community structure of JG117 vs. CB9 (Fig. 2D). This demonstrates that temperature and varietal differences significantly influence microbial community dynamics in rice.

To identify OTUs associated with cold stress, we analyzed the bacterial diversity in seeds and seedlings of the cold-tolerant JG117 and cold-sensitive CB9 rice varieties, under both cold stress (4°C) and non-stress (26°C) conditions. Our analysis revealed that, while bacterial abundance in the seeds of JG117 did not significantly differ from CB9

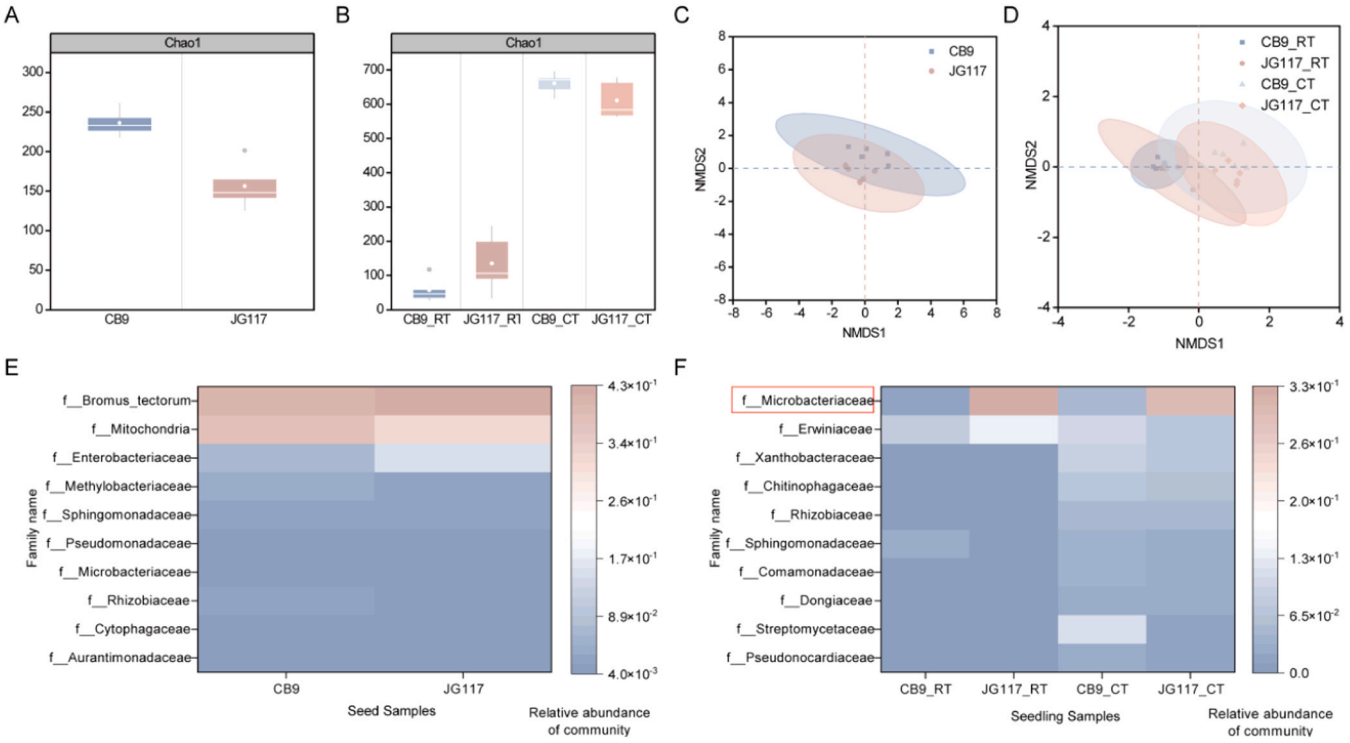

Fig. 2. Microbial diversities of rice seeds and seedlings of the cold-tolerant JG117 and cold-sensitive CB9 varieties under different temperature treatments. (A) Alpha diversity indices (Chao1) of microbial species richness and community diversity in seeds of JG117 and CB9. (B) Alpha diversity indices (Chao1) of seedlings of JG117 and CB9 grown under normal temperature conditions (RT) and cold stress (CT). (C) Beta diversity analysis by non-metric multidimensional scaling (NMDS) to evaluate differences in microbial community structure between JG117 and CB9 seeds. (D) Beta diversity analysis by NMDS for seedlings of JG117 and CB9, showing the effects of the indicated temperature treatments and rice varieties on microbial community structure. (E) Taxonomic heatmap analysis of the most abundant OTUs and their taxonomies in seeds of JG117 and CB9. (F) Taxonomic heatmap analysis of the most abundant OTUs and their taxonomies in seedlings of JG117 and CB9 under different temperature treatments. CB9_CT: cold-treated CB9 seedlings, JG117_CT: cold-treated JG117 seedlings, CB9_RT: room temperature-treated CB9 seedlings, JG117_RT: room temperature-treated JG117 seedlings.

seeds (Fig. 2E), there was a markedly higher prevalence of the Microbacteriaceae family in JG117 seedlings compared to CB9 seedlings under both temperature conditions (Fig. 2F). Specifically, under cold stress, the abundance of Microbacteriaceae in JG117 seedlings was approximately 531.93 % higher than in CB9 (0.2946 vs. 0.0466), and under room temperature conditions, the abundance in JG117 was approximately 1579.71 % higher than in CB9 (0.3255 vs. 0.0194). The consistent and significant presence of Microbacteriaceae in the cold-tolerant variety JG117, compared to the cold-sensitive CB9, led us to hypothesize that this bacterial family might contribute substantially to cold tolerance. Consequently, our study focused on the specific role of Microbacteriaceae in enhancing cold tolerance in rice.

3.3. Microbiological characterization of the Microbacteriaceae family

To harness the cold-tolerance traits of JG117 and the effects of seed endophytic microbes throughout the rice growth cycle, endophytic microbes were isolated from JG117 seeds under low-temperature conditions. Consequently, we isolated and purified 178 bacterial strains from JG117 seeds incubated at 10°C. By 16S rRNA sequencing and comparison with the GenBank database via NCBI Blast, we identified five unique strains of the Microbacteriaceae family, excluding duplicates and known plant pathogens: M14 (*Curtobacterium albidum*), M15 (*Microbacterium testaceum*), M21 (*Curtobacterium oceanosedimentum*), M73 (*Curtobacterium pusillum*), and M123 (*Curtobacterium citreum*).

We evaluated the ability of these strains to survive under low-temperature conditions. M15 (*Microbacterium testaceum*) exhibited superior cold tolerance compared to other members of the Microbacteriaceae family (Fig. 3A). This highlights the tolerance of M15 to cold conditions.

Next, we focused on the ability of M15 to enhance the cold tolerance of the cold-sensitive rice variety CB9. We soaked seeds during germination and irrigated seedling roots with bacterial suspensions. After 7 days of cultivation at normal temperature (26°C), followed by a 5-day cold stress treatment at 4°C and a 7-day recovery at 26°C, M15-treated seedlings showed a survival rate of 88.89 ± 11.11 %, significantly higher than the control group (44.44 ± 11.11 %), effectively doubling the resilience of CB9 under cold stress conditions (Fig. 3B). These findings suggest that M15 can enhance the cold tolerance of rice.

We assessed the plant growth-promoting potential of M15 and other strains by examining their biochemical characteristics. M15 showed outstanding IAA production (43.27 ± 4.25 mg/mL), which may explain its promotion of plant growth (Fig. 3C). Additionally, M15 showed the highest phosphate solubilization capacity among the tested strains (173.68 ± 1.53 mg/L), indicating its potential to enhance nutrient availability (Fig. 3D). All five Microbacteriaceae strains were capable of siderophore production (Fig. 3E), which is likely linked to their enhancement of plant growth.

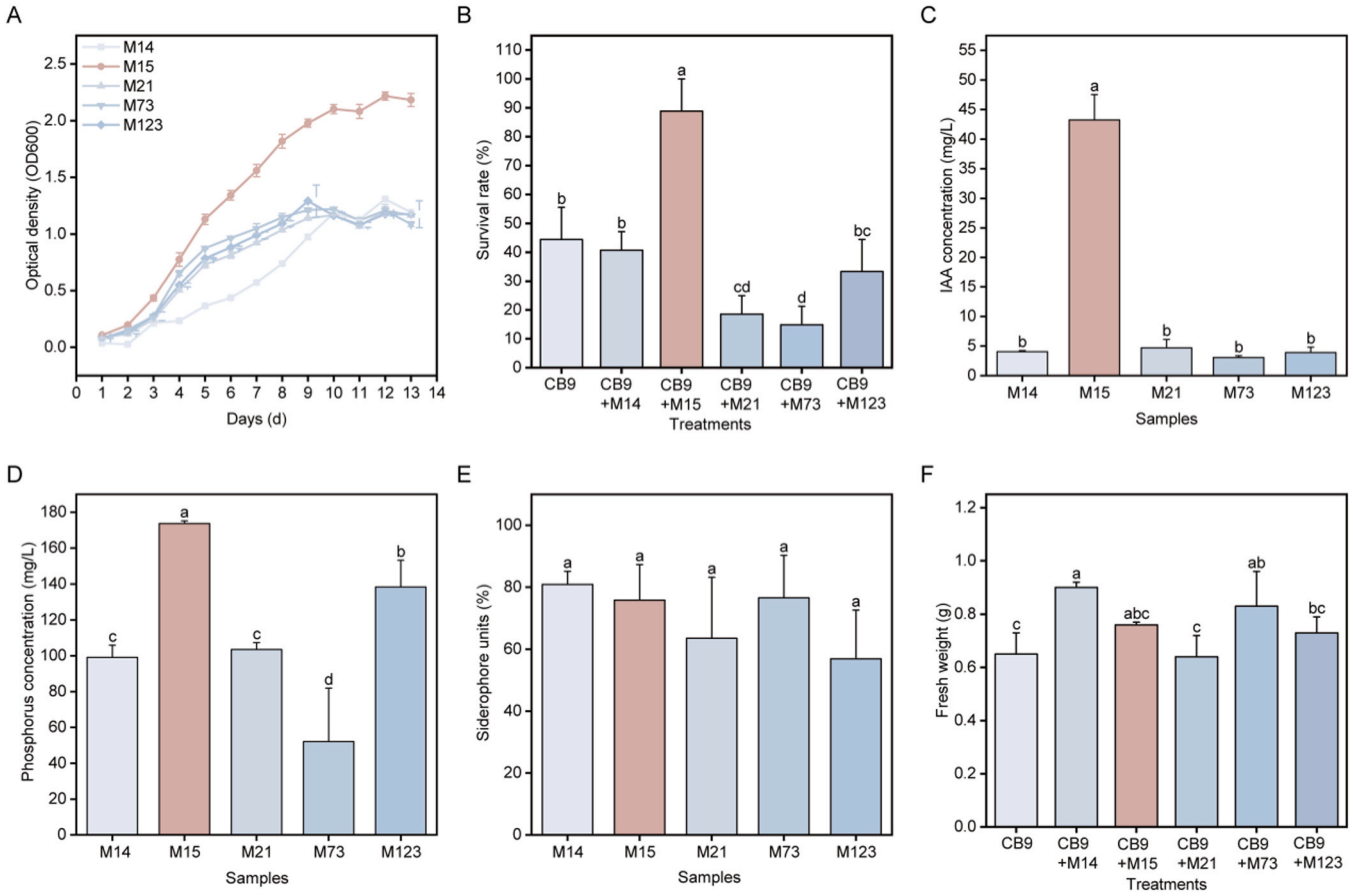

Fig. 3. Characterization of Microbacteriaceae and their effects on rice growth and cold tolerance. (A) Growth curves of Microbacteriaceae strains (M14, *Curtobacterium albidum*; M15, *Microbacterium testaceum*; M21, *Curtobacterium oceanosedimentum*; M73, *Curtobacterium pusillum*; and M123, *Curtobacterium citreum*) cultured at low temperature (4℃). (B) Survival rate of CB9 seedlings treated with the indicated Microbacteriaceae strains after cold stress at 4℃ and recovery at 26℃. (C) Indole-3-acetic acid (IAA) production by Microbacteriaceae strains. (D) Phosphate solubilization by Microbacteriaceae strains. (E) Siderophore production by Microbacteriaceae strains. (F) Fresh weights of CB9 rice seedlings treated with the indicated Microbacteriaceae strains under normal temperature conditions (26℃). CB9: CB9 rice without inoculation, CB9+M14: CB9 rice with M14 inoculation, CB9+M15: CB9 rice with M15 inoculation, CB9+M21: CB9 rice with M21 inoculation, CB9+M73: CB9 rice with M73 inoculation, CB9+M123: CB9 rice with M123 inoculation. Bar values are from three independent biological replicates. Mean values labeled with the same letter were not significantly different at $p < 0.05$.

Under low-temperature conditions (4°C), M15 maintained a high level of IAA production (39.94 ± 0.74 mg/mL), although this was slightly reduced compared to normal conditions. In terms of phosphate solubilization, M15 showed some inhibition under cold stress, with a solubilization level of 29.18 ± 2.16 mg/L, but still outperformed the other strains, indicating its consistent ability to promote plant growth across different environmental conditions. Siderophore production by M15 was also reduced under cold stress, a trend observed in the other strains as well (Fig. S1). These results suggest that M15 and other Microbacteriaceae strains retain their plant growth-promoting potential under cold stress, making them valuable candidates for enhancing plant resilience in cold climates.

Finally, we measured the fresh weight of three-leaf stage seedlings of CB9 rice grown under normal temperature conditions (26°C). Although M15 did not show the highest fresh weight (0.93 ± 0.01 g), it significantly increased the biomass of rice seedlings compared to the control (0.59 ± 0.08 g), highlighting its ability to promote plant growth (Fig. 3F). *M. testaceum* was selected for further investigation because of

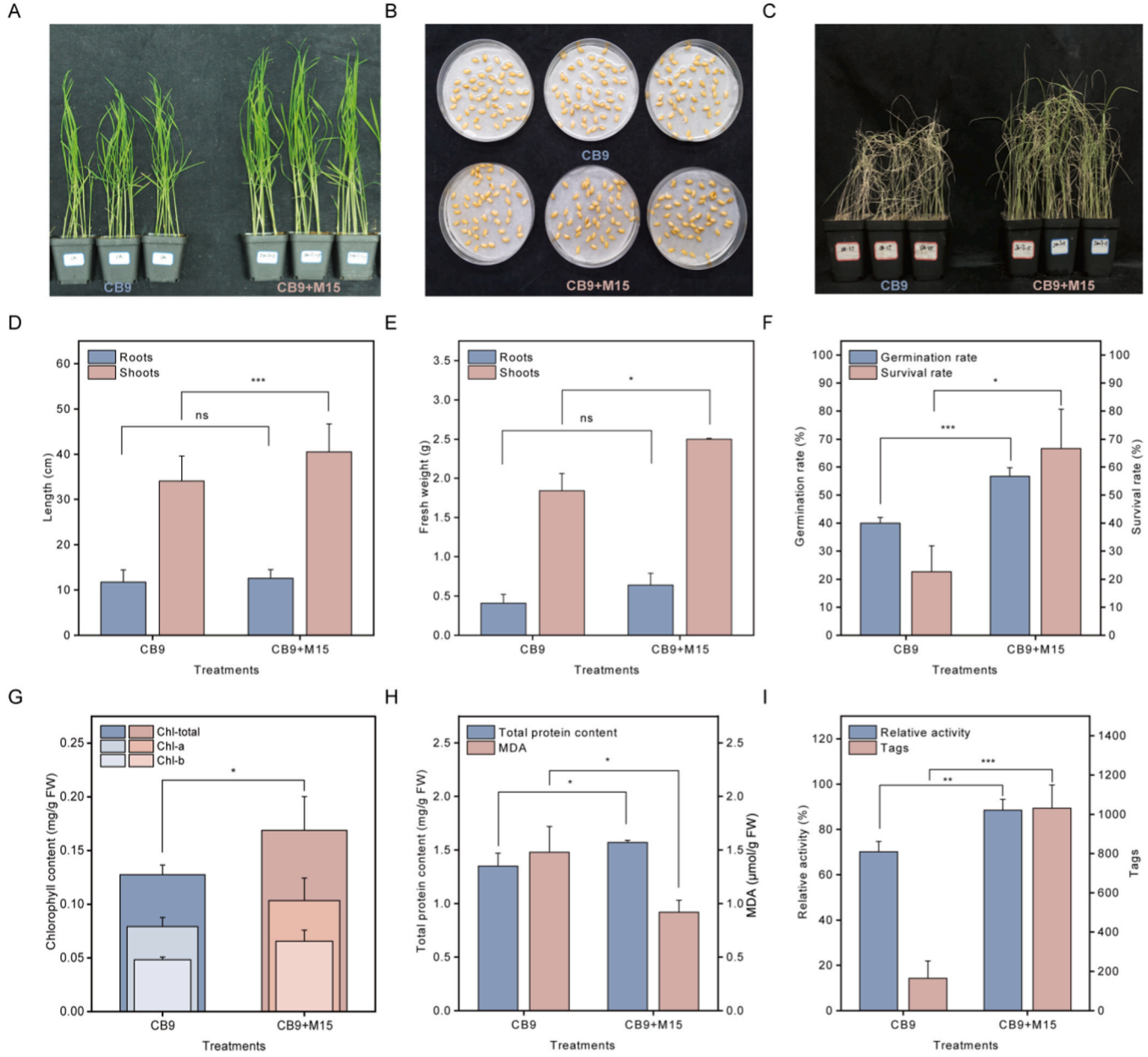

Fig. 4. Colonization by M15 of rice and effect on rice germination, growth, and cold tolerance. (A) Growth of rice seedlings under normal temperature conditions (26℃) with the indicated inoculations. (B) Germination of rice under low-temperature conditions (14℃) with the indicated inoculation. (C) Recovery growth of rice after low-temperature stress (4℃) with the indicated inoculations. (D) Plant height and root length of rice under normal temperature conditions (26℃) with the indicated inoculations. (E) Fresh weight of stems and roots of rice under normal temperature conditions (26℃) with the indicated inoculations. (F) Germination rate of rice seeds under low-temperature conditions (14℃) with the indicated inoculations. Survival rate of rice seedlings after low-temperature stress (4℃) and recovery at normal temperature (26℃) with the indicated inoculations. (G) Chlorophyll content in rice leaves after low-temperature stress with the indicated inoculations. (H) Total protein content in rice leaves after low-temperature stress with the indicated inoculations. MDA content in rice leaves after low-temperature stress with the indicated inoculations. (I) Relative catalase (CAT) activity in rice leaves after low-temperature stress with the indicated inoculations. Abundance of Microbacteriaceae microbes in rice with different inoculation treatments. CB: CB9 rice without inoculation treatment, CB9+M15: CB9 rice with M15 inoculation treatment. Chl-a, chlorophyll a; Chl-b, chlorophyll *b*, Chl-total, total chlorophyll; MDA, malondialdehyde. Data are from three independent biological replicates. Mean values labeled with the same letter are not significantly different at $p < 0.05$.

its exceptional cold tolerance, promotion of plant growth, and potential to enhance the tolerance of rice to cold stress.

3.4. M15 Enhances the cold tolerance and growth of rice seedlings

To evaluate the ability of M15 to enhance the growth and cold tolerance of CB9 rice, we investigated its effect on seedling growth, cold tolerance, physiological and biochemical characteristics, and colonization.

M15 promoted the growth and cold tolerance of CB9 rice seedlings cultivated in vermiculite with bacterial solution applied during germination and growth (Fig. 4). M15-treated CB9 seedlings (CB9+M15) showed increased shoot and root lengths (6.5 and 0.79 cm) and fresh weights (0.66 and 0.23 g), suggesting promotion of rice growth (Figs. 4A, 4D, 4E). Additionally, it ignificantly improved the germination rate at 14°C from 40 % to 56.67 % and doubled the survival rate of seedlings after cold stress from 22.67 % to 66.67 % (Fig. 4F), highlighting its ability to enhance cold tolerance (Figs. 4B and 4C).

M15 inoculation increased the contents of chlorophyll a by 30.45 %, chlorophyll *b* by 35.78 %, and total chlorophyll by 32.45 % (Fig. 4G). It also increased the total protein content in rice leaves by 16.31 %, reduced the MDA content by 37.76 % (Fig. 4H), and increased catalase activity by 26.15 % (Fig. 4I). These changes suggest that M15 reduced oxidative stress and promoted growth under cold conditions.

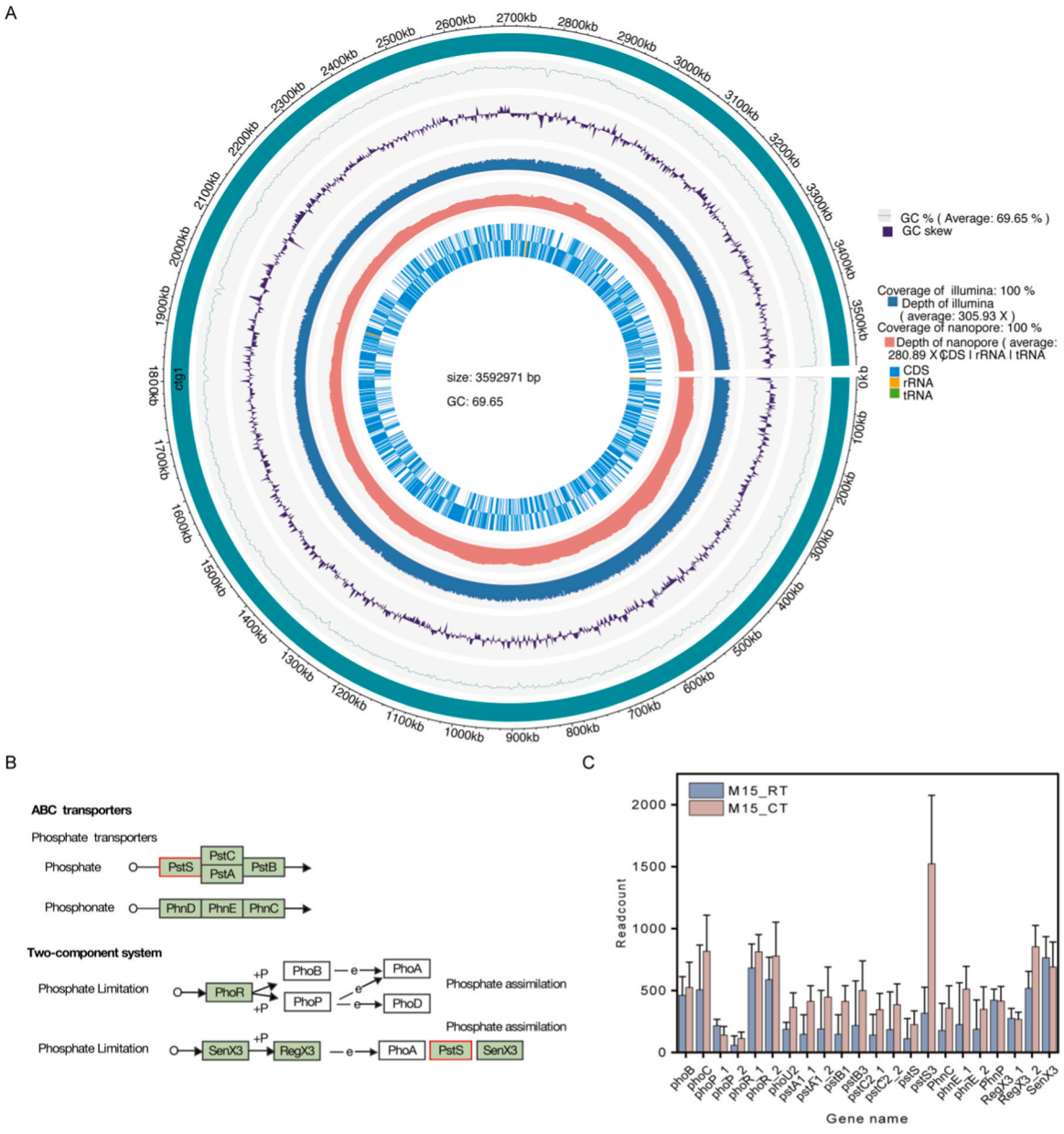

Fig. 5. Genomic and transcriptomic analyses of M15 under the indicated temperature conditions. (A) Genomic landscape of M15 revealed by circular genome mapping. This plot provides a view of the genome's general characteristics, including its structure, variations in GC content, sequencing coverage, and distribution of coding and non-coding RNA regions. (B) KEGG pathway analysis of phosphate transport genes in M. testaceum M15 under different temperature conditions. The transcriptional profiles of phosphate transport-related genes were analyzed under normal (37°C) and low (4°C) temperatures. Genes highlighted in green boxes are actively involved in the phosphate transport pathway. Genes with a white background are those present in the pathway but without significant changes in expression. Upregulated genes are in red. (C) Expression levels of genes in the phosphate transport system (Pho and Pst) in M15 under normal (37°C) and low temperature (4°C) conditions. M15_RT, M15 cultured at normal temperature (37°C). M15_CT, M15 cultured at low temperature (4°C). Data are from three independent biological replicates.

Colonization was confirmed by the significant increase in the Microbacteriaceae population in treated CB9 seeds grown in 1/2 MS agar from 165 ± 88.10 OTU tags in untreated seeds to 1031 ± 118.20 OTU tags (Fig. 4I), indicating successful microbial establishment and potential alteration of endophytic microbial dynamics in rice.

Additionally, EPS production by M15 was assessed under different temperature conditions to understand its role in enhancing cold tolerance. At 37°C, M15 produced an average of 52.40 ± 8.18 mg/L of EPS. Under cold stress at 4°C, the average EPS production significantly increased to 90.88 ± 12.59 mg/L (Figs. S2). This substantial rise in EPS production under low-temperature conditions suggests that M15 boosts its protective functions by enhancing EPS production, which likely contributes to its ability to support plant survival and growth in cold environments.

3.5. Genomic and transcriptomic insights into the mechanisms by which M15 enhances the cold tolerance and growth of rice

We performed genomic sequencing of *M. testaceum* M15. The entire sequence (NCBI GenBank accession number CP143563, BioProject number PRJNA1069338, BioSample number SAMN39615697) includes a single circular chromosome with a total length of 3592,971 base pairs (bp). The genome had 3414 predicted genes, including 3344 protein-coding sequences, 49 tRNA genes, 3 each of 23S rRNA, 16S rRNA, and 5S rRNA genes, 1 tmRNA, and 1 miscellaneous RNA, with an average G+C content of 69.95 % (Fig. 5A).

Genome annotation and functional analysis of M15 revealed the mechanisms by which it adapts to harsh environments, enhancing plant growth and improving cold tolerance. GO, KEGG, and COG analyses identified genes crucial for cellular communication, material transport, and the stress response, as well as metabolic pathways involved in carbohydrate and amino acid metabolism, membrane transport, and energy conversion (Figs. S3–S5).

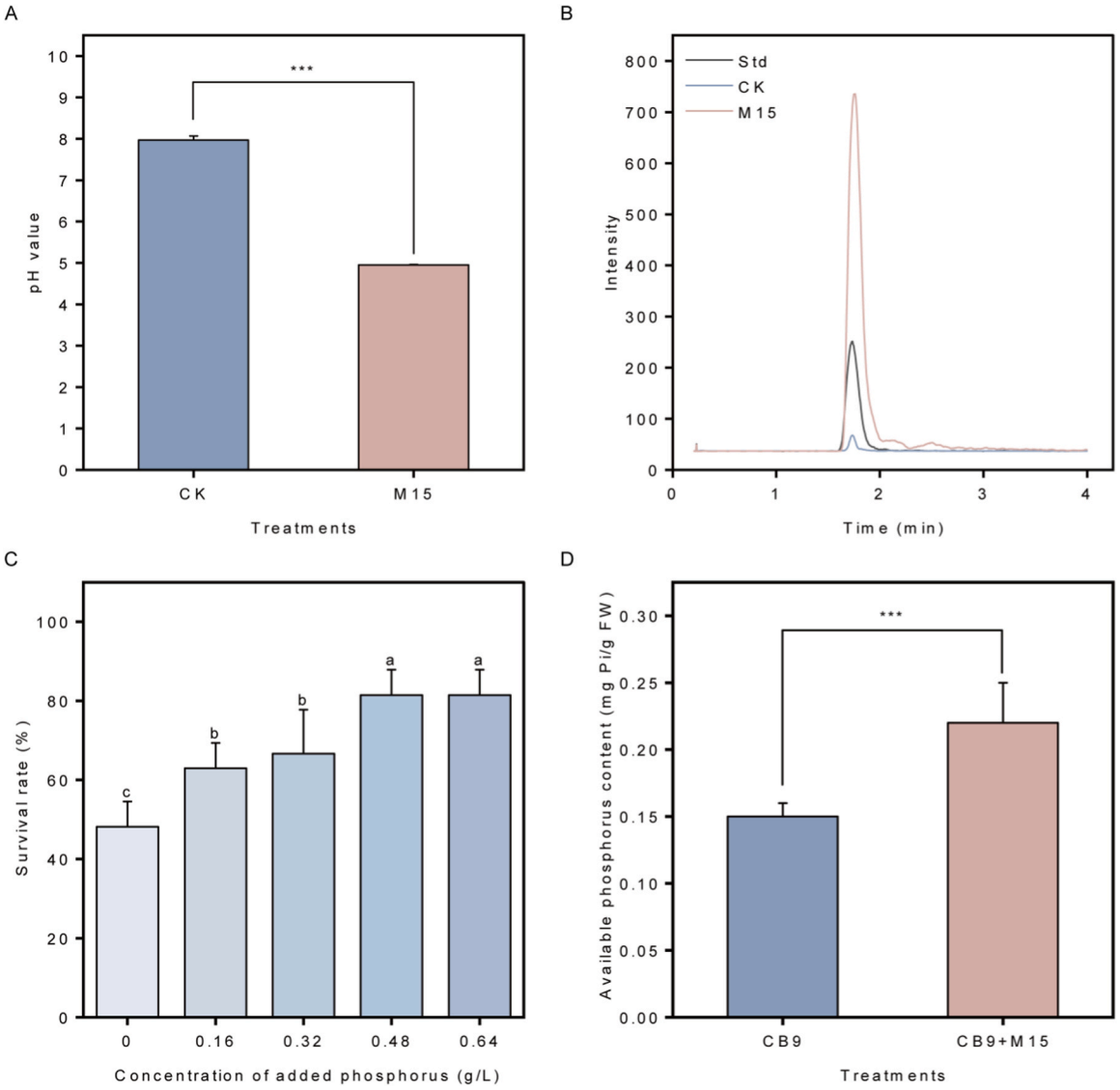

Fig. 6. Phosphorus solubilization by M15 and its role in enhancing the cold tolerance of rice. (A) pH of NBRIP medium after 1 day of incubation without and with M15 inoculation. (B) Gluconic acid content in the supernatant of NBRIP medium after 1 day of incubation without and with M15 inoculation, as measured by LC-QQQ. (C) Survival rate of rice seedlings after cold stress (4℃) and recovery at normal temperature (26℃) with different levels of exogenous phosphorus supplementation. (D) Available phosphorus content of rice leaves at the seedling stage without and with M15 inoculation. CK, NBRIP medium without inoculation; M15, NBRIP medium inoculated with M15; STD, gluconic acid standard; CB9, CB9 rice without inoculation; CB9+M15, CB9 rice inoculated with M15. Data are from three independent biological replicates. Mean values labeled with the same letter are not significantly different at $p < 0.05$.

Genomic analysis identified numerous genes in M15 associated with low-temperature adaptability. These included those encoding cold-shock proteins, heat-shock proteins, molecular chaperones, antioxidant enzymes, and osmoprotectants. Additionally, genes whose products modify the cell membrane and cell wall, as well as those responsible for Exopolysaccharides (EPS) production, were found (Table S1). Moreover, M15 had genes that promote plant growth, such as those responsible for the synthesis of IAA, phosphate solubilization, and siderophore production (Table S2). Additionally, genes that facilitate plant colonization were identified (Table S2), aligning with the establishment of M15 in rice and its significant effect on the Microbacteriaceae community.

Next, we conducted transcriptomic analysis of M15 cultured under normal (37°C) and low-temperature (4°C) conditions. Differential gene expression and KEGG pathway analysis revealed that genes associated with the phosphate regulon (Pho system) and phosphate-specific transport system (Pst system) were upregulated under cold conditions (Figs. 5B, 5C). Details of the functions of the phosphate transport genes are listed in Table S3. These data highlight the importance of phosphate transport genes in enhancing the cold tolerance of M15, thus contributing to improved phosphorus uptake and stress resilience in rice.

3.6. M15 enhances phosphorus availability and cold tolerance in rice

To understand the contribution of phosphorus solubilization by M15 to the enhanced cold tolerance of rice, we investigated its phosphorus solubilization and its effect on the resilience of rice to cold conditions.

M15 demonstrated significant phosphorus solubilization in NBRIP medium, lowering the pH from 7.97 ± 0.10 in the control to 4.95 ± 0.01 after 24 h (Fig. 6A). Furthermore, gluconic acid, a phosphate solubilizing agent, was detected only in culture medium inoculated with M15 (Fig. 6B), implicating it in phosphate solubilization.

The influence of exogenous phosphorus on cold tolerance was evaluated by adding phosphorus at different concentrations and assessing survival post-cold stress. The addition of phosphorus at 0.64 g/L significantly increased the survival rate of rice seedlings from 48.15 ± 6.42 % in the control to 81.48 ± 6.42 % (Fig. 6C), underscoring the role of phosphorus in enhancing cold tolerance.

Inoculation of M15 increased the available phosphorus content of rice leaves from 0.15 ± 0.01 mg Pi/g FW in the control to 0.22 ± 0.03 mg Pi/g FW (Fig. 6D), which may be linked to the M15-mediated enhancement of plant growth and cold tolerance.

4. Discussion

The effect of global climate change on crop yields necessitates innovative strategies to enhance the stress tolerance of plants. We identified a cold-tolerant endophytic bacterium, *M. testaceum* M15, from seeds of a cold-tolerant rice variety, JG117. M15 enhanced the cold tolerance and growth of a cold-sensitive rice variety, CB9, underscoring the potential of microbial inoculants for sustainable agriculture. These findings suggest strategies for improving crop resilience to cold stress, especially in temperate and high-altitude regions, thereby contributing to food security.

Significant differences in microbial composition were observed in rice seedlings with varying cold tolerance, particularly in the abundance of Microbacteriaceae. Therefore, certain microbial communities modulate the cold tolerance of rice. Rice genotype and environment significantly influence microbial composition, and our findings support this, indicating that microbial community changes can impact host growth and stress tolerance (Walitang et al., 2018; Tian et al., 2023). The observed differences in microbial communities among rice varieties highlight the importance of genotype-specific microbial interactions when developing microbial inoculants (Cui et al., 2019). In plants, Microbacteriaceae modulate plant responses to stress conditions. Our findings provide insight into the changes in the Microbacteriaceae community and their role in enhancing the cold tolerance of rice (Patel et al., 2022).

From the cold-tolerant rice variety JG117, *Microbacterium testaceum* M15, a member of the Microbacteriaceae family, exhibited notable growth-promoting traits at both room temperature (37°C) and low temperature (4°C), although its efficiency varied depending on environmental conditions. Despite reduced production of certain growth-promoting factors at lower temperatures, M15 effectively enhanced plant growth and stress resilience, demonstrating its adaptability across different temperatures. This adaptability suggests that M15 has the potential to be utilized under various environmental conditions, making it a versatile tool for enhancing crop resilience in different climates. Inoculation of rice with *M. testaceum* M15 significantly promoted the growth, seed germination, and seedling survival of the cold-sensitive CB9 under cold stress.

M15 increased the root length, plant height, and biomass, indicating improved nutrient accumulation and growth. Inoculation of M15 enhanced rice growth by improving photosynthesis and protein synthesis (Kakar et al., 2016; Tiryaki et al., 2019). It also alleviated the effect of cold stress on rice by reducing the MDA content and increasing CAT activity, thereby promoting antioxidant and anti-lipid peroxidation activities (Pramanik et al., 2017; Xie et al., 2019; Zhou et al., 2021). These findings are consistent with previous reports of the effectiveness of microbial inoculants to promote the growth and tolerance to abiotic stresses of plants (Mishra et al., 2012; Wu et al., 2019; Kour and Yadav, 2023a). This study's novelty lies in the use of M15, a cold-tolerant endophytic bacterium from rice seeds, to improve a cold-sensitive rice variety. This cross-variety inoculation leverages different hosts' genetic backgrounds to adjust and reconstruct the host microbiome, which has potential for agricultural applications (Berg and Raaijmakers, 2018).

The results of multi-omics analysis suggested the mechanisms by which M15 enhances the cold tolerance and growth of rice (Fig. 7). Whole-genome sequencing of M15 revealed multiple key genes related to cold adaptation, including those encoding cold-shock proteins, heat-shock proteins, and molecular chaperones (Shen et al., 2021; Dasila et al., 2022; Singh et al., 2022). Antioxidant-related genes are crucial for reducing oxidative damage (Jeong et al., 2000; Saikolappan et al., 2011; Guan et al., 2017). Genes involved in carotenoid production aid in the scavenging of reactive oxygen species (ROS), thereby reducing oxidative damage caused by cold stress (Dasila et al., 2022). Genes responsible for producing compatible solutes and cell protectants such as betaine, trehalose, tetrahydropyrimidine, mannitol, and proline provide protection to cells (Wemekamp-Kamphuis et al., 2004; Csonka Laszlo and Leisinger, 2007; Guan et al., 2017). Moreover, genes that regulate the cell membrane and cell wall structure, along with those responsible for exopolysaccharide synthesis and transport, contribute to cellular protection (Methé et al., 2005; Dasila et al., 2022; Kumar et al., 2022). The presence of genes related to IAA synthesis in M15 indicates that it can directly affect rice growth via endogenous hormone pathways (Puranik et al., 2022). The genes associated with phosphate solubilization and siderophore production underscore the role M15 in enhancing nutrient availability and promoting plant health (Xing et al., 2021; Silva et al., 2023; Wang et al., 2023). These findings provide insight into the role of M15 in supporting plant survival and growth under challenging conditions. They also highlight the potential of endophytic bacteria to boost crop performance, laying a foundation for applications in agricultural biotechnology (Kushwaha et al., 2020). As a microbial inoculant, M15 has considerable potential for enhancing crop adaptation to environmental stress.

Transcriptomic analysis under cold-stress conditions showed significant upregulation of genes involved in phosphate transport systems. This highlights an adaptive mechanism whereby M15 enhances rice phosphate uptake and utilization by improving phosphate solubilization. In addition, the available phosphorus content in rice leaves was increased by inoculation of M15, and enhanced phosphate availability via microbial solubilization is crucial for maintaining growth and

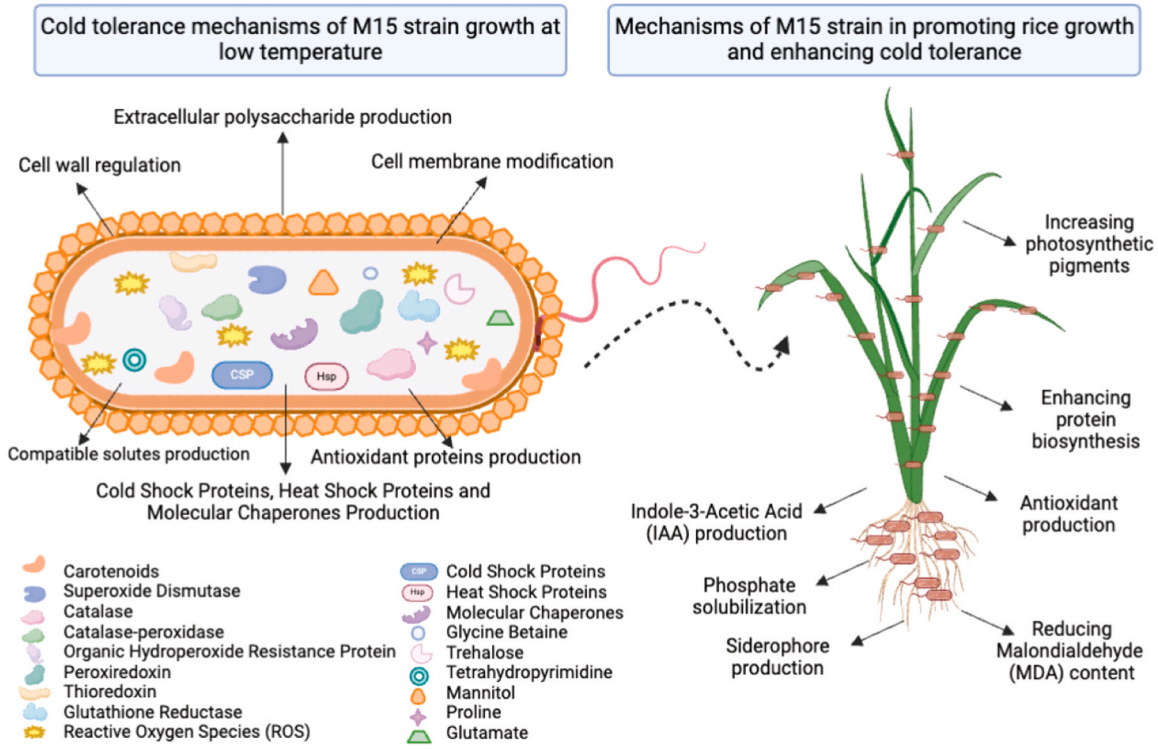

Fig. 7. Mechanisms of cold tolerance and growth promotion in rice by M15 under low-temperature conditions. The cold tolerance mechanisms of M15 are the production of cold-shock proteins, heat-shock proteins, and molecular chaperones; modulation of cell wall properties; cell membrane modification; synthesis of compatible solute protectants; and generation of antioxidant proteins to mitigate oxidative damage caused by cold stress. The growth-promoting and cold-resistance mechanisms of M15 in rice involve the production of indole-3-acetic acid (IAA); phosphate solubilization; siderophore production; increased levels of photosynthetic pigments; enhancement of protein biosynthesis; production of antioxidants; and reduction of malondialdehyde (MDA) content. Created with BioRender.com.

metabolic activities under cold stress, thereby promoting rice growth and cold tolerance (Li-gang et al., 2012). This study provides insights into the phosphate solubilization mechanism of M15, which is associated with medium acidification and the production of organic acids, particularly gluconic acid. Improved phosphate utilization significantly enhances rice cold tolerance (Fitriatin et al., 2022). These findings underscore the role of microbial phosphate solubilization in mitigating the adverse effects of cold stress during the seedling stage. *M. testaceum* M15, as a phosphate-solubilizing microbial inoculant, can potentially reduce phosphate fertilizer use, decrease agricultural runoff and water pollution, improve ecosystem health, and promote sustainable agricultural development, offering potential environmental benefits.

Our research underscores the importance of seed endophytic microbes such as M15 for enhancing plant growth and stress tolerance under cold stress, suggesting them to be vital contributors to agricultural sustainability and resilience to stress. However, field trials under different environmental conditions and with various rice varieties are needed to validate the results and ensure broad applicability. Future research should also explore the interactions between M15 and other microbial components in the rice microbiome to evaluate the dynamics of microbial inoculants within rice microbiomes and identify potential synergistic or antagonistic relationships that may affect their efficacy. Insight into these complex interactions will suggest novel strategies for enhancing crop resilience to environmental stresses.

5. Conclusion

Our findings highlight the potential of seed endophytic microbes, particularly *M. testaceum* M15, to enhance rice growth and cold tolerance. There was a strong association between Microbacteriaceae and the cold tolerance of rice varieties. *M. testaceum* M15, isolated from the cold-tolerant rice variety JG117, promoted rice growth and development under cold stress by mitigating the negative effects on germination and seedling growth.

M15 enhanced cold tolerance by boosting photosynthesis, protein synthesis, and antioxidant defenses while reducing lipid peroxidation. We identified genes associated with cold adaptation and growth enhancement, which may explain the ability of M15 to promote plant growth and health. Transcriptomic analysis indicated upregulation of genes associated with phosphate transport systems under cold conditions, likely an adaptive mechanism for improving phosphate absorption and utilization. The colonization by M15 of rice seedlings and its effect on the endophytic microbial community highlight its potential as a microbial inoculant.

There is a need for field trials under diverse environmental conditions to explore the interactions between M15 and other members of the rice microbiome. This would provide insight into the dynamics of microbial inoculants within plant microbiomes and identify synergistic relationships that enhance their efficacy. Such information would advance agricultural biotechnology, enhance crop resilience to environmental challenges and promote sustainable agricultural practices. Our findings offer a sustainable and environmentally friendly strategy to combat cold stress in rice, contributing to food security in temperate and high-altitude regions, which are vulnerable to the effects of climate change.

CRediT authorship contribution statement

Ningfeng Wu: Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Huiying Luo:** Supervision, Project administration, Funding acquisition. **Chunyi Zhang:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Bin Yao:** Supervision, Resources, Project administration, Funding acquisition. **Jian Tian:** Writing – review & editing, Supervision, Resources,

Project administration, Conceptualization. **Jintong Zhao:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Pierre Delaplace:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Ligang Hou:** Supervision, Resources, Methodology. **Xiaoqing Liu:** Supervision, Methodology, Formal analysis, Conceptualization. **Feifei Guan:** Supervision, Formal analysis. **Guoshun Xu:** Methodology, Investigation. **Wei Zhang:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127908.

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