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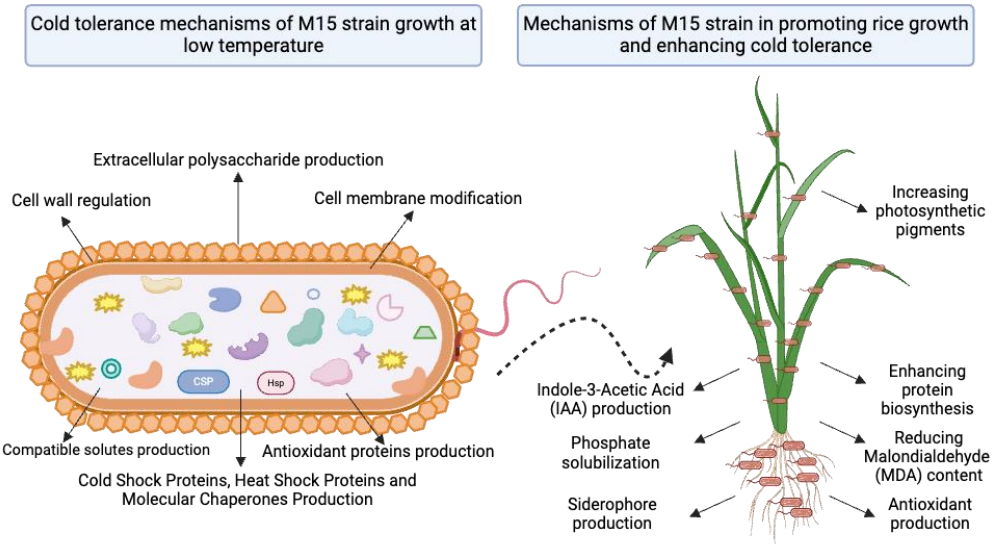
Rice growth promotion and cold stress alleviation by an endophytic bacterium *Microbacterium testaceum* M15 isolated from rice seed



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Rice growth promotion and cold stress alleviation by an endophytic bacterium *Microbacterium testaceum* M15 isolated from rice seed

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Abstract

Jintong Zhao (2025). "Rice growth promotion and cold stress alleviation by an endophytic bacterium *Microbacterium testaceum* M15 isolated from rice seed" (PhD Dissertation in English).

Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege.

199 pages, 39 figures, 4 tables.

Abstract:

Rice (*Oryza sativa* L.) is one of the world's most essential food crops. With global climate change intensifying, cold stress has become a significant environmental factor that negatively affects rice growth, yield, and quality, thus posing a major threat to food security. Enhancing rice cold tolerance is crucial not only for maintaining growth and yield under cold conditions but also for ensuring its quality. Recent advances in plant-microbe interaction studies have provided new insights and technical strategies for enhancing crop resilience to environmental stresses. Microbial communities play a crucial role in supporting plant growth, aiding in environmental adaptation, and enhancing stress tolerance. Despite this, their impact on rice cold tolerance has not been extensively studied.

This study systematically investigates the relationship between microbial communities and rice cold tolerance using a multifaceted experimental approach. Firstly, the microbial communities in the seeds and seedlings of two rice varieties, the cold-tolerant JG117 and cold-sensitive CB9, were compared. The results revealed that cold-tolerant varieties harbored more diverse and abundant microbial communities, with a relative high level of abundance of Microbacteriaceae family. This finding supports the hypothesis that microbial communities play a vital role in rice cold tolerance and suggests a potential link between microbial communities and rice stress tolerance, providing a foundation for future research.

Subsequently, microorganisms were isolated from the seeds of the cold-tolerant variety JG117, with a focus on the Microbacteriaceae family. *Microbacterium testaceum* M15 was found to exhibit significant plant growth-promoting properties, such as indole-3-acetic acid (IAA) production, phosphate solubilization, and siderophore production. Inoculation of the cold-sensitive variety CB9 with the *M. testaceum* M15 strain through seed soaking and root drenching resulted in notable improvements in growth and enhanced cold tolerance under cold stress.

Additionally, the study conducted a comprehensive analysis to understand how *M. testaceum* M15 enhances cold tolerance in rice. M15-inoculated rice seedlings showed higher chlorophyll content, total protein levels, and catalase activity, while malondialdehyde content was reduced, indicating that *M. testaceum* M15 helps mitigate oxidative damage and improve cold tolerance. Genomic and transcriptomic analyses revealed that *M. testaceum* M15 aids rice in adapting to low temperatures by solubilizing phosphate, enhancing phosphate transport, and inducing the

expression of cold tolerance-related genes. Additionally, *M. testaceum* M15 enhances the activity of the rice antioxidant system, further improving cold tolerance. Inoculating *M. testaceum* M15 during the rice booting stage increased cold tolerance and improved agronomic traits at maturity, such as plant height, panicle length, 1,000-grain weight, and filled grain number, while reducing the number of unfilled grains. Microbial community analysis showed that *M. testaceum* M15 not only colonized the endophytic environment but also optimized the microbiome, promoting growth and enhancing cold tolerance in rice.

This study provides new theoretical insights into the intrinsic relationship between rice cold tolerance and microbial communities. It also offers a novel perspective on the application of microbial technology in agriculture. As extreme climate events associated with global climate change become more frequent, microorganisms offer a sustainable solution to enhance crop tolerance to adverse conditions, thereby improving food crop yield and quality. The findings of this study provide a foundation for potential microbe-based approaches to enhance rice cold tolerance and support the sustainable advancement of agriculture.

Keywords: Rice, Cold tolerance, Microbial community, *Microbacterium testaceum*, Agricultural sustainability

Résumé

Jintong Zhao (2025). "Promotion de la croissance du riz et atténuation du stress dû au froid par une bactérie endophyte, *Microbacterium testaceum* M15 isolée des graines de riz" (Thèse de doctorat en anglais).

Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

199 pages, 39 figures, 4 tableaux.

Résumé:

Le riz (*Oryza sativa* L.) est l'une des cultures alimentaires essentielles au niveau mondial. Avec l'intensification du changement climatique mondial, le stress dû aux températures basses est devenu un facteur environnemental majeur affectant négativement la croissance, le rendement et la qualité du riz, représentant ainsi une menace importante pour la sécurité alimentaire. Améliorer la tolérance du riz au froid est crucial non seulement pour maintenir la croissance et le rendement sous des conditions de basse température, mais aussi pour assurer sa qualité. Les avancées récentes dans les études sur l'interaction plante-microbe ont fourni de nouvelles perspectives et des stratégies techniques pour améliorer la résilience des cultures face aux stress environnementaux. Les communautés microbiennes jouent un rôle clé dans le soutien de la croissance des plantes, l'adaptation environnementale et l'amélioration de la tolérance aux stress. Cependant, leur impact sur la tolérance du riz au froid n'a pas été largement étudié.

Cette étude examine de manière systématique la relation entre les communautés microbiennes et la tolérance au froid du riz en utilisant une approche expérimentale multifacette. Tout d'abord, les communautés microbiennes des graines et des plantules de deux variétés de riz, la variété tolérante au froid JG117 et la variété sensible au froid CB9, ont été comparées. Les résultats ont révélé que les variétés tolérantes au froid abritaient des communautés microbiennes plus diversifiées et abondantes, avec une relative abondance particulièrement élevée de la famille Microbacteriaceae. Cette découverte soutient l'hypothèse selon laquelle les communautés microbiennes jouent un rôle crucial dans la tolérance au froid du riz et suggère un lien potentiel entre les communautés microbiennes et la tolérance du riz aux stress, fournissant ainsi une base pour les recherches futures.

Ensuite, des micro-organismes ont été isolés des graines de la variété tolérante au froid JG117, en mettant l'accent sur la famille Microbacteriaceae. *Microbacterium testaceum* M15 a montré des propriétés significatives de promotion de la croissance des plantes, telles que la production d'acide indole-3-acétique (IAA), la solubilisation du phosphate et la production de sidérophores. L'inoculation de la variété sensible au froid CB9 avec la souche *M. testaceum* M15 par trempage des graines et trempage des racines a entraîné des améliorations notables de la croissance et une meilleure tolérance au froid sous stress thermique.

De plus, l'étude a mené une analyse approfondie pour comprendre comment *M.*

testaceum M15 améliore la tolérance au froid du riz. Le riz inoculé a montré une teneur plus élevée en chlorophylle, des niveaux totaux de protéines et une activité de catalase accrus, tandis que la teneur en malondialdéhyde a été réduite, indiquant que *M. testaceum* M15 aide à atténuer les dommages oxydatifs et à améliorer la tolérance au froid. Les analyses génomiques et transcriptomiques ont révélé que *M. testaceum* M15 aide le riz à s'adapter aux basses températures en solubilisant le phosphate, en améliorant le transport du phosphate et en induisant l'expression de gènes liés à la tolérance au froid. De plus, *M. testaceum* M15 améliore l'activité du système antioxydant du riz, ce qui accroit la tolérance au froid. L'inoculation de *M. testaceum* M15 pendant le stade de montaison du riz a augmenté la tolérance au froid et amélioré les traits agronomiques à maturité, tels que la hauteur des plantes, la longueur des panicules, le poids de 1000 grains et le nombre de grains remplis, tout en réduisant le nombre de grains vides. L'analyse de la communauté microbienne a montré que *M. testaceum* M15 n'a pas seulement colonisé l'environnement endophyte du riz, mais a également optimisé le microbiome du riz, favorisant la croissance et améliorant la tolérance au froid.

Cette étude fournit de nouvelles perspectives théoriques sur la relation intrinsèque entre la tolérance au froid du riz et les communautés microbiennes. Elle offre également une nouvelle perspective sur l'application de la technologie microbienne en agriculture. À mesure que les événements climatiques extrêmes associés au changement climatique mondial deviennent plus fréquents, les micro-organismes offrent une solution durable pour améliorer la tolérance des cultures aux conditions défavorables, améliorant ainsi le rendement et la qualité des cultures alimentaires. Les résultats de cette étude fournissent une base pour les approches microbiennes potentielles visant à améliorer la tolérance au froid du riz et à soutenir l'avancement durable de l'agriculture.

Mots-clés: Riz, Tolérance au froid, Communauté microbienne, *Microbacterium testaceum*, Durabilité agricole

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List of abbreviations

ROS: reactive oxygen species
GA: gibberellins
IAA: indole-3-acetic acid
ABA: abscisic acid
ATP: adenosine triphosphate
SOD: superoxide dismutase
APX: ascorbate peroxidase
CAT: catalase
POD: peroxidase
SA: salicylic acid
PGPB: plant growth-promoting bacteria
AFPs: antifreeze proteins
ACC: 1-aminocyclopropane-1-carboxylate
AHL: N-acyl homoserine lactone
P: phosphorus
PSMs: phosphate-solubilizing microorganisms
EPS: exopolysaccharide
PSB: phosphate-solubilizing bacteria
PGPMs: plant growth-promoting microorganisms
CKs: cytokinins
MDA: malondialdehyde
PGPR: growth-promoting rhizobacteria
CSPs: cold shock proteins
HSPs: heat shock proteins
NBRIP: National Botanical Research Institute's Phosphate
PGP: plant growth-promoting

Chapter 1

General introduction

This chapter lays the foundation for the entire thesis, providing the research background, objectives, research roadmap, and an overview of the structure of the thesis, helping readers understand the significance and scope of the study. As the first chapter, it introduces the core research topics, such as rice growth promotion, cold tolerance, and the role of endophytic microorganisms, setting the stage for the subsequent literature review and experimental chapters.

1.1 Context

Rice (*Oryza sativa* L.) is one of the most important staple crops worldwide, providing essential food security for billions of people (Mohidem et al., 2022). However, rice is highly sensitive to environmental changes throughout its growth cycle, particularly to cold stress. This stress significantly affects rice growth, development, and yield, especially in high-latitude or climate-volatile regions, where it severely limits the geographical scope of rice cultivation.

Cold stress impacts the entire rice growth cycle but is most pronounced during the seedling and panicle initiation stages. During the seedling stage, low temperatures slow growth, reduce photosynthetic capacity, lead to excessive accumulation of reactive oxygen species (ROS), and hinder root development, which weakens the plant's overall stress tolerance (Wang et al., 2021a). In the panicle initiation stage, low temperatures impair grain filling and maturation, significantly reducing final yield and quality. These negative effects are particularly evident in regions with unstable climates (Li et al., 2022). As global climate change intensifies, the increasing frequency of extreme weather events further exacerbates the threat of cold stress to rice production. In response to this challenge, developing effective strategies to enhance rice cold tolerance has become a key focus of modern agricultural research.

Traditional agricultural approaches to mitigate cold stress mainly include varietal improvement, optimized agronomic practices, and the application of modern agricultural technologies (Cao et al., 2022; El-Refaee et al., 2024). However, these methods face numerous challenges in practice. For instance, varietal improvement requires long development periods and substantial costs, and is constrained by factors such as climate conditions, planting regions, and production cycles. Furthermore, although modern protective cultivation techniques can alleviate cold stress in the short term, their high economic costs limit their large-scale implementation. Consequently, attention has gradually shifted towards plant endophytic microorganisms, an area that holds significant potential.

Endophytic microorganisms are a group of microbes that reside within plant tissues without causing disease, including bacteria and fungi (Nair and Padmavathy, 2014). Studies have shown that endophytic microorganisms can promote plant nutrient uptake, enhance antioxidant enzyme activity, and secrete plant growth regulators, significantly improving plant stress tolerance and growth performance (Watts et al., 2023). Therefore, developing bio-based formulations using endophytic microorganisms is considered a promising strategy to enhance plant stress tolerance and promote sustainable agriculture.

Different rice varieties exhibit significant variations in the composition and functional characteristics of their endophytic microbiomes due to differences in genetic background and growth environment (Khanal et al., 2024; Liu et al., 2024). These microbial communities not only determine the rice plant's ability to adapt to cold stress but also directly influence its growth phenotype. By comparing the endophytic microbiomes of different varieties, key microorganisms with outstanding functional characteristics can be identified as potential targets for microbial

formulation development, offering new solutions for improving rice stress tolerance and optimizing crop management.

Seed endophytic microorganisms have become a significant focus in recent plant-microbe interaction research. As the starting point of the plant life cycle, the endophytic microbiome of seeds exhibits high genetic stability and can influence the phenotype and stress tolerance of subsequent generations through vertical transmission. For instance, certain endophytic microorganisms within seeds can enhance seedling stress tolerance and vitality during early growth stages, providing a natural protective barrier for plant development (Wang and Zhang, 2023). However, research on the interaction between seed endophytic microorganisms and rice cold tolerance remains insufficient. Further investigation into the role of seed endophytic microorganisms in enhancing rice cold tolerance will not only help elucidate the underlying mechanisms but also provide a theoretical foundation for the development of agricultural technologies focused on seed treatments.

Additionally, Microbacteriaceae, a family of microorganisms with diverse functional traits and ecological adaptability, has garnered attention in recent years. These microorganisms are widely distributed in ecosystems such as soil, plants, and water bodies (Vasilenko et al., 2018). Studies have shown that they play an important role in promoting plant growth and enhancing stress tolerance (Walitang et al., 2017; Wang et al., 2021c). However, research on the role of Microbacteriaceae under cold stress is limited, and their potential in improving plant cold tolerance remains underexplored.

Based on this, the present study focuses on *Microbacterium testaceum* M15, an endophytic bacterium isolated from cold-tolerant rice seeds, to systematically investigate its role in promoting rice growth under cold stress and the underlying mechanisms. Through genomic and transcriptomic analysis, this study will reveal the molecular mechanisms by which the M15 strain enhances rice cold tolerance and promotes growth, as well as explore its potential applications in agricultural production. The findings of this research will not only provide theoretical support for a deeper understanding of plant-endophytic microbe interactions but also offer new ideas for the development of green agricultural technologies and economically efficient bioformulations, contributing to the sustainable development of rice cultivation in cold regions.

1.2 Research objectives

This study aims to investigate the mechanisms by which *Microbacterium testaceum* M15 enhances rice growth and cold tolerance, and to evaluate its potential applications in sustainable agriculture. The specific objectives are as follows:

(1) Explore the growth performance and endophytic microbial diversity of cold-tolerant rice varieties

Compare the growth performance and cold tolerance differences between the cold-tolerant rice variety JG117 and the cold-sensitive variety CB9 under both normal and cold conditions, providing baseline data for understanding rice's cold

tolerance mechanisms. Additionally, analyze the diversity, community structure, and distribution characteristics of the endophytic microbiomes in the seeds and seedlings of the two rice varieties to investigate their potential relationship with cold tolerance.

(2) Screen and identify endophytic microorganisms in cold-tolerant rice seeds

Isolate endophytic microorganisms from the seeds of the cold-tolerant rice variety JG117 and screen for key strains with cold tolerance and plant growth-promoting potential, providing microbial resources for further study.

(3) Measure the effects of the selected strain on growth and cold tolerance in cold-sensitive rice

Investigate the effects of the *Microbacterium testaceum* M15 strain on the growth and cold tolerance of the cold-sensitive CB9 rice under both normal and cold conditions. Analyze its physiological and biochemical mechanisms to provide evidence for understanding the strain's function.

(4) Analyze the genomic and transcriptomic characteristics of the M15 strain to reveal its molecular mechanisms

Through genomic and transcriptomic analyses, identify the key genes and functional characteristics of the M15 strain related to cold adaptation, plant growth promotion, and stress tolerance. Further uncover the molecular mechanisms underlying its effects.

(5) Investigate the cold tolerance effects of the M15 strain during the reproductive stage of rice and its impact on grain microbial communities

Evaluate the impact of the M15 strain during the reproductive stage of rice on physiological performance, yield-related traits, and the diversity of grain-associated microbial communities. This will provide scientific evidence for its potential in agricultural applications.

1.3 Research outline

This dissertation is divided into eight chapters, organized as follows:

Chapter 1: General introduction

This chapter provides an overview of the research background, objectives, research roadmap, and the structure of the thesis, establishing the foundation for understanding the significance and scope of the study.

Chapter 2: Literature review

This chapter discusses the impact of cold stress on rice, the role of seed endophytic microorganisms in stress tolerance, the research progress on Microbacteriaceae microorganisms, and the potential of phosphate-solubilizing microorganisms in sustainable agriculture.

Chapter 3: Growth performance and endophytic microbial community characteristics of cold-tolerant and cold-sensitive rice varieties

This chapter evaluates the cold tolerance and growth performance of the cold-tolerant rice variety JG117, and analyzes the diversity and structural

characteristics of the endophytic microbiomes in the seeds and seedlings of both the cold-tolerant variety JG117 and the cold-sensitive variety CB9 under normal and cold conditions.

Chapter 4: Isolation, identification, and functional characterization of endophytic microorganisms from seeds of cold-tolerant rice varieties

This chapter presents the isolation and identification of Microbacteriaceae endophytic microorganisms from the seeds of the cold-tolerant rice variety JG117 and evaluates the functional characteristics of these microorganisms.

Chapter 5: Evaluation and mechanistic analysis of *Microbacterium testaceum* M15 in promoting rice growth and enhancing cold tolerance

This chapter investigates the physiological and biochemical responses of CB9 seedlings to inoculation with *Microbacterium testaceum* M15 under normal and cold conditions, and analyzes its potential as a plant growth-promoting bacterium.

Chapter 6: Genomic and transcriptomic insights into the molecular mechanisms underlying the role of *Microbacterium testaceum* M15 in enhancing rice cold tolerance

This chapter explores the genomic characteristics of the M15 strain, focusing on key genes related to cold adaptation and plant growth promotion. The integration of transcriptomic analysis is used to uncover the molecular mechanisms underlying M15's effects under different temperature conditions.

Chapter 7: Evaluation of the application potential of *Microbacterium testaceum* M15 in rice cultivation under cold conditions

This chapter evaluates the effects of M15 on the cold tolerance and agronomic traits of CB9 rice during the reproductive stage and investigates its regulatory role on the diversity and composition of grain-associated microbial communities, highlighting its potential in agricultural applications.

Chapter 8: General discussion, conclusion and perspectives

This chapter summarizes the main findings of the study, discusses their significance in the context of sustainable agriculture, and suggests potential application directions for microbial inoculants, including *Microbacterium testaceum* M15.

1.4 Research roadmap

To achieve the scientific objectives of this study, a systematic research roadmap has been developed (see Figure 1.1), which is divided into the following five main phases:

Phase 1: Growth performance and microbial diversity analysis of cold-tolerant rice varieties

Two rice varieties with significant differences in cold tolerance—JG117 (cold-tolerant variety) and CB9 (cold-sensitive variety)—will be cultivated under both normal and cold conditions. By comparing their growth performance, physiological indicators, and cold tolerance, baseline data will be provided for

subsequent research. Additionally, the diversity and structural characteristics of the endophytic microbiomes in the seeds and seedlings of these two varieties under different temperature conditions will be analyzed to explore the potential impact of endophytic microorganisms on cold tolerance.

Phase 2: Isolation and screening of endophytic microorganisms

Endophytic microorganisms will be isolated from the seeds of the cold-tolerant rice variety JG117. The microorganisms will be classified and identified using 16S rRNA gene sequencing technology, and a phylogenetic tree will be constructed. Special attention will be paid to the Microbacteriaceae family, and through functional validation, key strains with significant cold tolerance and plant growth-promoting potential will be selected.

Phase 3: Impact of *Microbacterium testaceum* M15 strain on rice seedling growth and cold tolerance

The selected key strain—*Microbacterium testaceum* M15—will be systematically evaluated for its effects on the seed germination rate, seedling growth, and cold injury survival rate of the CB9 rice variety. Physiological and biochemical analyses will be conducted to explore the mechanisms by which the M15 strain promotes rice growth and enhances cold tolerance.

Phase 4: Genomic and transcriptomic analysis of *Microbacterium testaceum* M15 strain

Through genome sequencing, the key functional genes of the M15 strain involved in cold adaptation, antioxidant regulation, and plant growth promotion will be analyzed. At the transcriptomic level, gene expression changes in M15 under cold stress conditions will be examined, and key metabolic pathways and biological mechanisms regulating cold tolerance will be identified.

Phase 5: Cold tolerance of *Microbacterium testaceum* M15 strain during the reproductive stage and regulation of grain microbial communities

The cold tolerance effect of the M15 strain during the reproductive stage of rice will be further investigated, and its impact on physiological performance, yield, and quality-related traits will be evaluated. Additionally, the composition and diversity changes of the grain-associated endophytic microbial communities in rice inoculated with M15 will be analyzed using high-throughput sequencing technology, to explore the colonization ability of M15 and its regulatory effects on the host microbiome.

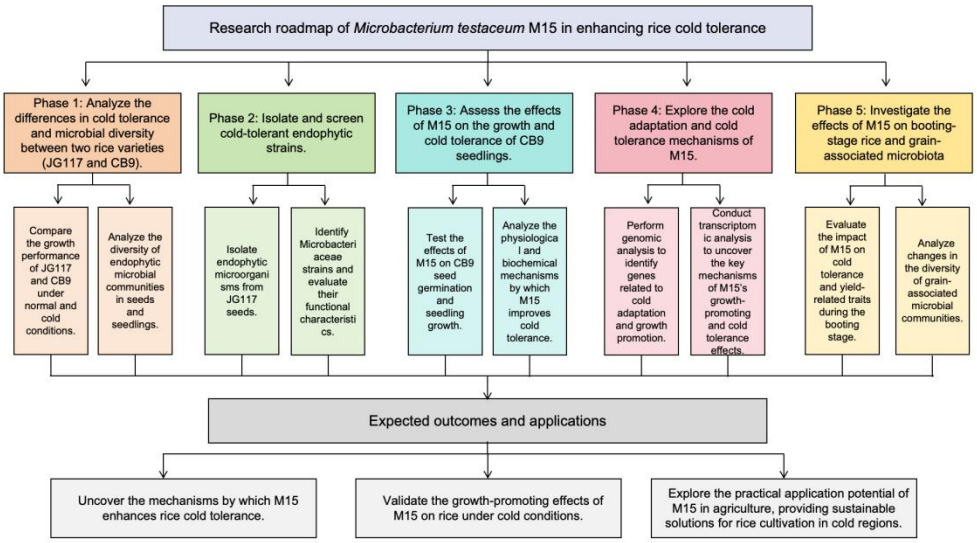

Figure 1.1 Research roadmap of the entire thesis. This figure outlines the research process used to achieve the study's objectives, divided into five main phases.

Chapter 2

Literature review

Building upon the background introduced in Chapter 1, this chapter delves further into existing literature regarding rice cold tolerance, the role of seed endophytic microorganisms in stress tolerance, and research progress on Microbacteriaceae microorganisms. The chapter discusses the effects of cold stress on rice and presents research advances on the potential of phosphate-solubilizing microorganisms in sustainable agriculture. By reviewing existing studies, this chapter identifies research gaps, laying the foundation for the experimental methods and research design in the subsequent chapters.

This chapter is adapted from the following published review article:

Zhao J, Yu X, Zhang C, Hou L, Wu N, Zhang W, Wang Y, Yao B, Delaplace P, Tian J. Harnessing microbial interactions with rice: Strategies for abiotic stress alleviation in the face of environmental challenges and climate change. Science of The Total Environment **2024**;912:168847. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.168847

2.1 Effects of cold stress on rice growth

Rice (*Oryza sativa* L.) is one of the most important staple crops globally, playing a crucial role in food security, particularly in Asia (Fukagawa and Ziska, 2019). Approximately half of the global population relies on rice as their primary source of calories, with the dependence being particularly significant in Asia (Hashim et al., 2024). China and India are the largest producers and consumers of rice worldwide, together accounting for more than 50% of the global rice production (Muthayya et al., 2014; Irantha, 2024). In addition, Vietnam, Thailand, the Philippines, Myanmar, Bangladesh and Indonesia are also key rice-producing and exporting countries. The rice production and per capita rice consumption of the eight Asian countries—China, India, Vietnam, Thailand, the Philippines, Myanmar, Bangladesh, and Indonesia—account for approximately 80% of the global total (Maraseni et al., 2018; Bin Rahman and Zhang, 2023). Rice is not only a staple in Asia but has also become an increasingly important food crop in some countries in Sub-Saharan Africa and Latin America.

Table 1.1 Projections of population in major rice producing and consuming countries in Asia, 1995 to 2025

Country	Population (mill.) 1995	Annual Growth Rate (% per year) 1995-2000	2020-2025	Projected Population (mill.) in 2025	Percent Increase1995 -2025
China	1199	0.9	0.5	1471	23
India	934	1.7	1.0	1370	47
Indonesia	192	1.4	0.8	265	38
Bangladesh	121	1.8	1.1	182	50
Vietnam	74.1	2.0	1.2	117	58
Thailand	60.5	1.3	0.7	80.8	34
Myanmar	46.8	2.1	1.1	72.9	56
Japan	125	0.3	-0.3	124	-1
Philippines	69.2	2.2	1.2	115	66
Rep. of Korea	44.8	0.8	0.3	52.9	18
Pakistan	130	2.7	1.6	243	87
Asia (excluding China)	2244	1.8	1.1	3389	51

Source: Rice production the Asia-pacific region: issues and perspectives. (https://www.fao.org/4/x6905e/x6905e04.htm)

As a typical temperate to tropical plant, rice exhibits optimal growth in warm and humid environments, with a suitable temperature ranging from 25℃ to 35℃ (Hussain et al., 2019). When the environmental temperature falls below 14℃ rice growth and development are significantly inhibited. This cold stress is especially detrimental during the seedling and flowering stages, potentially leading to growth stagnation, yield reduction, or even total crop failure. In recent years, with the intensification of climate change, the frequency of extreme climate events such as cold waves and frosts has significantly increased, posing an escalating threat to rice production. This issue is particularly severe in high-latitude and high-altitude regions, where cold stress has become a major limiting factor for rice production.

The effects of cold stress on rice are reflected in multiple aspects, including growth development, physiological metabolism, and molecular regulation. Cold stress leads to elevated reactive oxygen species (ROS) levels, lipid peroxidation of cell membranes, and a decline in photosynthetic efficiency, thus inhibiting rice growth performance (Guo et al., 2022). Furthermore, cold stress also interferes with the balance of endogenous hormones and gene expression, affecting the cold tolerance and adaptability of rice (Khan et al., 2023c). Therefore, in-depth studies on the mechanisms of cold stress in rice are of great significance for developing cold-tolerant varieties and improving rice cultivation techniques.

2.1.1 Geographical distribution and climatic requirements of rice cultivation

Rice is widely cultivated across tropical and temperate regions, although its production density and cultivated area vary depending on geographic and climatic conditions (Cordero-Lara, 2020). The distribution of rice cultivation exhibits significant latitude differences, typically requiring warm climates with sufficient rainfall for optimal growth. Rice growth demands suitable climatic conditions, with temperature being especially sensitive. The most favorable temperature range for rice growth is typically between 25℃ and 35℃, although temperature requirements vary depending on the region and growth stage. Rice cultivation methods and climate demands differ based on geographic latitude. In tropical and subtropical regions, the growth cycle of rice is longer, with warm climates and higher optimal growth temperatures. In contrast, rice cultivation in temperate regions has a shorter growing season, with harsher climate conditions, particularly the threat posed by low winter temperatures or spring frosts.

Globally, the primary rice cultivation areas are concentrated in Asia, which is not only the center of global rice production but also the major consumer region. For example, the rice cultivation areas and production capacities of China, India, Indonesia, Vietnam, and Thailand are crucial for global food security. In addition, in some countries of Sub-Saharan Africa and Latin America, rice is becoming an increasingly important food crop, although production scales and technological levels are still far below those of Asia (Adjao and Staatz, 2015).

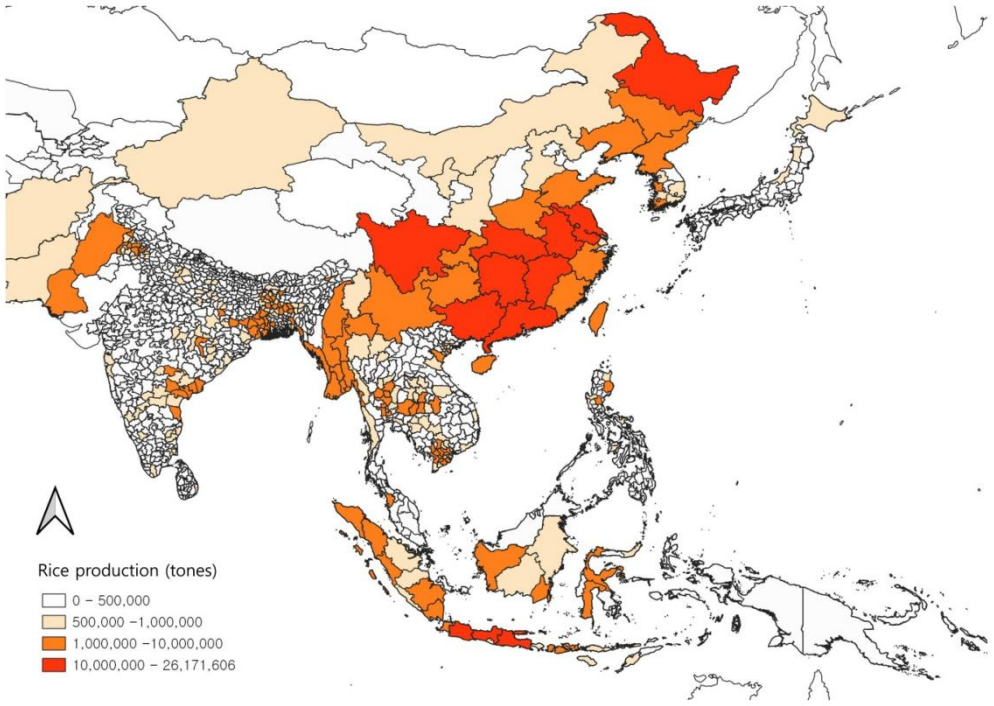

Figure 2.1 Map of rice cultivation areas with productions (Hwang et al., 2022).

2.1.1.1 Characteristics of rice cultivation in Asia

Rice cultivation in Asia is diverse, encompassing tropical, subtropical, and temperate regions with varying ecological types and cultivation methods. South Asia and Southeast Asia primarily have tropical and subtropical climates, where monsoon rainfall provides sufficient water and stable high temperatures for rice growth. In these regions, the rice growth cycle is relatively long, typically exceeding 150 days. This long-cycle cultivation system relies on stable monsoon climates, and with proper management, yields are relatively stable. However, climate change, which has led to uncertainties in rainfall patterns and frequent extreme climate events, such as typhoons and floods, poses new threats to rice production.

In contrast, rice cultivation in East Asia is primarily found in temperate regions, such as China, Japan, and South Korea. Rice production in these regions heavily relies on irrigation systems and modern agricultural technologies, with more intensive management practices. Due to the higher latitude, the rice growth cycle in East Asia is shorter, typically 90 to 120 days, with a narrower planting window. cold stress significantly impacts production. In Japan's Hokkaido and China's northeastern provinces, spring low temperatures or late frosts can cause seedling growth stagnation or even frost damage. Additionally, during the booting or ripening

stages, cold waves may significantly reduce grain setting rates.

2.1.1.2 Characteristics of rice cultivation outside Asia

Although Asia is the center of global rice production and consumption, the rice cultivation area in sub-Saharan Africa and Latin America has significantly increased in recent decades. The rice-growing environments and production systems in these regions differ markedly from those in Asia, primarily constrained by local climatic and agricultural conditions (Rodenburg and Saito, 2022). In Africa, rice cultivation is primarily rainfed, and its productivity is highly dependent on the timing and distribution of rainfall. However, due to the high uncertainty of rainfall, rice yields in these regions fluctuate considerably, especially in the arid or semi-arid areas of sub-Saharan Africa (Balasubramanian et al., 2007).

In high-altitude regions such as Ethiopia and Rwanda, rice cultivation is limited by cold stress. Due to large diurnal temperature variations and low temperatures, the growing season for rice is extended, leading to lower yields. Similarly, in the Andean region of Latin America, rice cultivation faces not only cold stress but also constraints related to water scarcity and soil infertility. In these regions, local improved rice varieties are often used, but their cold tolerance and productivity still require further improvement.

2.1.1.3 Cold stress challenges in Northeast China

In China, rice cultivation is widespread across a variety of climatic zones, ranging from tropical to temperate regions. The tropical and subtropical areas in southern China typically offer favorable climatic conditions, whereas the northern temperate regions, especially Northeast China, face significant challenges from cold stress (Saud et al., 2022). Northeast China experiences a typical high-latitude temperate monsoon climate, with unique rice-growing conditions. The region has long, cold winters with an average annual temperature ranging from 2℃ to 6℃. Spring temperatures rise late, and autumn cooling occurs early, leading to a short growing season. Although the summer is warm, late frost and spring cold waves pose serious threats to rice growth and development. Despite these challenges, the rice cultivation area in Northeast China has rapidly expanded in recent years, becoming an important commodity grain production base in the country. However, cold stress, such as spring low temperatures and late frost, remains a key limiting factor for rice production in the region.

Spring low temperatures and late frost significantly delay seed germination and seedling growth, sometimes causing seedling death. Additionally, late frost occurring during the flowering or grain-filling stages can dramatically reduce grain setting rates, affecting grain filling speed and 1,000-grain weight. Studies have shown that rice cultivation in Northeast China requires the use of cold-tolerant local varieties and specific management strategies, such as adjusting planting times, employing cold acclimation techniques, and improving water and fertilizer management (Dong et al., 2023). Nevertheless, extreme cold events can still cause significant yield losses, presenting a long-term challenge to ensuring food security in the country.

2.1.2 Effects of cold stress on different growth stages of rice

Rice growth is highly sensitive to temperature variations, and different stages of its growth cycle have significantly different temperature requirements. For example, during germination, rice requires a temperature range of 20℃ to 30℃ to ensure rapid seed germination and uniform seedling emergence. During the seedling stage, stable high temperatures (around 25℃ to 30℃) are necessary to promote rapid root and shoot growth. During the flowering stage, temperatures around 30℃ are more favorable for pollination and grain setting. In the maturation stage, the optimal temperature range is typically between 22℃ and 30℃. Temperatures below 20℃ significantly delay the grain filling process, resulting in insufficient grain plumpness, which lowers the 1,000-grain weight and overall yield.

Cold stress is a complex environmental stress that affects rice growth and development through a series of physiological and molecular changes. The effects of low temperature on rice are not only manifested in physical and morphological changes but also involve complex regulatory mechanisms at the physiological and metabolic levels (Zhang et al., 2014). cold effects on rice occur through both direct and indirect mechanisms. Direct mechanisms include the inhibition of cellular metabolism and energy generation, manifested as a decrease in enzyme activity, impaired respiration, and reduced photosynthetic efficiency. Indirect mechanisms involve the induction of ROS accumulation, leading to lipid peroxidation of cell membranes and damage to membrane integrity. Moreover, low temperatures can alter rice gene expression patterns, affecting the activation and function of cold tolerance-related genes. cold stress is one of the main abiotic stresses affecting rice growth, with profound impacts across all growth stages. These effects are observed in plant morphological development, physiological metabolism, molecular regulation, as well as the final yield and quality. Cold stress influences rice throughout its entire life cycle, affecting germination, seedling, flowering, and maturation stages (Thakur et al., 2010; Singh, 2017; Shi et al., 2022). The sensitivity of each growth stage to cold stress differs, and the specific effects vary, influencing multiple aspects of plant growth, development, and physiological metabolic processes.

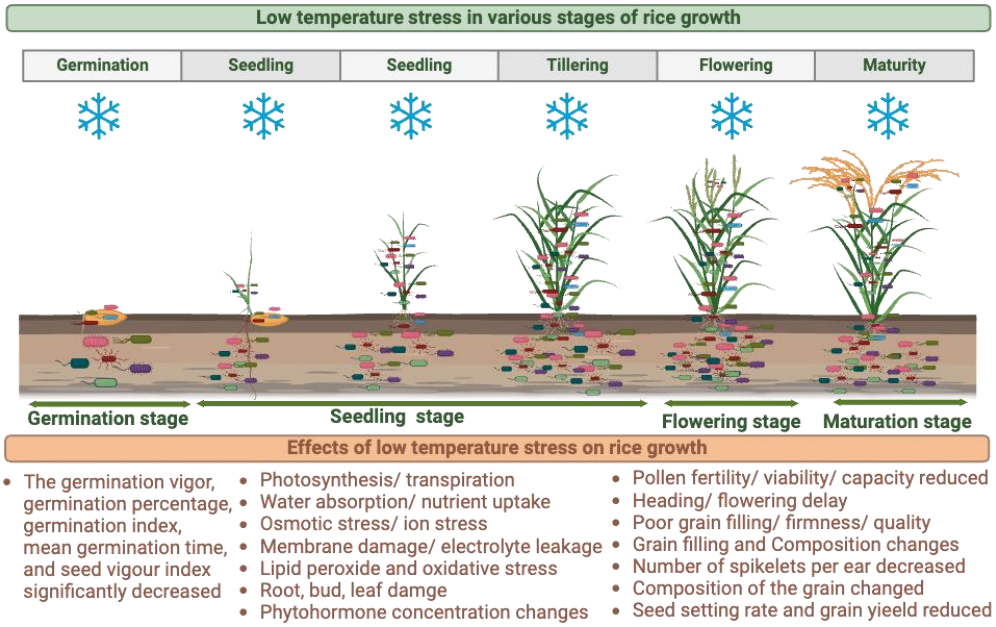

Figure 2.2 Cold stress in various stages of rice growth and effects of cold stress on rice growth. Created with BioRender.com.

2.1.2.1 Effects of cold stress on rice germination

Germination is the starting point of the rice life cycle, directly determining the plant's growth potential and final yield. However, the germination stage is particularly sensitive to environmental stresses. Cold stress during the germination period mainly affects physiological metabolism and reduces enzyme activity, leading to decreased germination rates and seedling vigor. Cold stress especially delays seed germination speed, lowers germination rates, and causes uneven seedling emergence and slow early root development. Research has shown that prolonged cold exposure significantly inhibits root tip cell division, reducing the length and number of both primary and secondary roots (Yildirim et al., 2020).

During seed germination, the regulation of endogenous hormones is particularly crucial for resisting cold stress. Studies have found that cold temperatures significantly lower the levels of gibberellins (GA) and indole-3-acetic acid (IAA) in seeds, while increasing the concentration of abscisic acid (ABA) (Colebrook et al., 2014). This hormonal imbalance directly affects cell division and elongation in seeds, leading to delayed germination. Furthermore, cold stress inhibits respiration, resulting in insufficient adenosine triphosphate (ATP) production, which in turn delays the elongation of the radicle and embryo, causing uneven germination and even seed decay. Additionally, cold stress impacts enzyme activity in seeds,

particularly the activity of amylases and proteases, hindering the breakdown and utilization of stored substances, which delays the supply of energy for cell division and root and shoot development, further suppressing seed germination. Cold stress also affects seed coat permeability, reducing the permeability of the seed cell membrane and its osmoregulatory capacity. This decreases the rate of water absorption by the seed, limiting seed expansion and cell division, and exacerbating the shortage of energy and material supply during the germination process.

2.1.2.2 Effects of cold stress on rice seedling stage

The seedling stage is a critical period in rice growth and development, during which rice plants are highly sensitive to cold stress. Cold stress significantly affects seedling morphology, leading to stunted growth, leaf chlorosis, and inhibited root development. The effects of cold stress on rice seedlings primarily manifest as a reduction in photosynthetic efficiency, damage to the cell membrane system, weakened root absorption capacity, and a decline in the plant's antioxidant abilities (Wang et al., 2021a).

Cold stress has a significant inhibitory effect on photosynthesis in rice seedlings. This limitation on photosynthesis directly results in reduced biomass accumulation and insufficient energy supply during the seedling period. These negative effects typically manifest as slowed growth or even death of the plant. In addition, cold stress accelerates leaf chlorosis and the degradation of chlorophyll, further limiting photosynthesis (Zhao et al., 2020).

Regarding the roots, cold stress significantly reduces the growth rate and absorption capacity of rice seedling roots. Research has shown that cold stress can markedly decrease the growth rate of rice seedlings and affect their root development (Fu et al., 2023). Under cold stress, the root activity of seedlings significantly declines, characterized by an extended root tip cell division cycle, and a reduction in both root length and root surface area. This root development impairment not only limits the seedling's ability to absorb water and nutrients, but it also exacerbates nutrient deficiencies in the aerial parts of the plant, further weakening the growth ability of the above-ground tissues. Meanwhile, cold stress induces an excessive accumulation of ROS, which damages the integrity of the cell membrane, affects the functional and structural stability of cells, and leads to increased electrolyte leakage and a decline in root absorption capacity. This damage is typically manifested as elevated ROS levels and more severe membrane lipid peroxidation, further reducing the efficiency of water and nutrient absorption by rice roots (Hsu and Hsu, 2019).

2.1.2.3 Effects of cold stress on rice flowering stage

The flowering stage is a critical period that determines rice yield, directly affecting its seed setting rate and overall productivity. Cold stress during this stage has a particularly significant impact. The negative effects of cold stress on rice during flowering are mainly reflected in the inhibition of pollen germination, abnormal pollen development, reduced pollen viability, hormone imbalance, obstacles to pollination and fertilization, and reduced seed formation rate, which ultimately

affects the seed setting rate and yield (Li et al., 2022). Especially under spring frost or cold wave conditions, rice pollen germination rate is significantly reduced, leading to an uneven flowering period and male flower pollination failure, thus reducing rice yield.

Cold stress significantly inhibits pollen formation and development, manifesting as a reduction in pollen grain numbers, uneven size, and structural abnormalities. Studies have shown that cold stress causes sucrose accumulation in the anther, while the activity of cell wall-bound acid invertase decreases, and starch is depleted in mature pollen grains (Oliver et al., 2005). Cold stress during the reproductive stage can disrupt meiosis, pollen viability, and fertilization, leading to infertility in plants (Zeng et al., 2017). Research indicates that cold stress causes an imbalance in hormones such as ABA and GA, as well as in the homeostasis of sugars (Huang et al., 2023).

In addition, cold stress reduces rice nitrogen uptake and nitrogen use efficiency, further decreasing leaf area index and net photosynthetic rate, ultimately reducing rice grain yield (Jia et al., 2019).

The damage caused by cold stress to rice during the reproductive growth stage is an extremely complex biophysical and biochemical process. Cold stress can affect rice's ROS, osmotic regulators (such as proline, glutathione, soluble proteins), and antioxidants (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) (Guo et al., 2022).

2.1.2.4 Effects of cold stress on rice maturation stage

Cold stress during the maturation stage of rice cannot be overlooked. The negative effects of cold stress during this stage mainly manifest as a reduction in the grain filling rate and a decline in grain quality, leading to a decrease in yield and market value (Siddik et al., 2019). Under low temperature conditions, the activity of starch synthase significantly decreases, resulting in an imbalance in the proportion of amylose and amylopectin in the grains. This leads to a noticeable decline in rice gel consistency and texture, negatively impacting its cooking quality and eating quality, which in turn reduces consumer acceptance and lowers market value (Ahmed et al., 2008; Hu et al., 2020; Pang et al., 2024b).

In addition, cold stress also has a significant impact on rice's disease resistance. Cold stress often weakens the plant's immune response, making it more susceptible to pathogen attacks, which affects both yield and quality. Under cold conditions, the moisture content on the surface of the grains is higher, making them more prone to fungal infections. Cold stress also extends the grain maturation period, increasing moisture content at harvest. This high moisture environment provides favorable conditions for pathogen invasion, increasing the risk of fungal contamination or disease during harvest, further compromising the storage stability and processing quality of the rice (Awuchi et al., 2021; Qiu et al., 2024).

Overall, cold stress, as a major abiotic stress, has a significant impact on the entire growth cycle of rice. Cold stress limits the growth potential of rice by inhibiting cell metabolism, disrupting photosynthetic efficiency, inducing the accumulation of ROS, and interfering with the balance of endogenous hormones and gene expression. At

the physiological level, cold stress leads to leaf chlorosis, impaired root development, and reduced biomass accumulation; at the metabolic level, cold stress inhibits the activity of key enzymes, reducing the synthesis of carbohydrates and proteins; at the molecular level, cold stress significantly alters the rice plant's tolerance and adaptability by regulating the expression of cold-tolerance genes. These complex mechanisms demonstrate that the negative impact of cold stress on rice is multidimensional and profound.

Meanwhile, in high-latitude regions and under the intensifying background of climate change, the frequency and intensity of cold stress are rising year by year. This not only increases the uncertainty of rice production but also poses a long-term threat to food security. For example, in regions such as Northeast China, Hokkaido in Japan, and the Andes Mountains in Latin America, cold stress has become a major factor limiting rice planting efficiency. In these areas, cold stress significantly weakens rice growth performance and yield formation, often by delaying seed germination, affecting pollen development, and reducing the grain filling rate. Therefore, reducing the impact of cold stress and improving rice cold tolerance has become key to increasing rice yield and ensuring food security.

2.2 Methods to improve rice cold tolerance

Cold stress has a profound impact on rice growth and production performance, and it is one of the major abiotic stresses limiting rice yield and quality. Throughout the entire growth cycle from germination to maturation, cold stress significantly weakens rice's growth potential through multiple mechanisms, such as inhibiting physiological metabolism, disrupting photosynthesis, inducing ROS accumulation, and interfering with gene expression. The effects of cold stress are particularly severe in high-latitude and high-altitude regions, where it not only threatens regional food security but also increases the uncertainty of agricultural production. Therefore, improving rice cold tolerance and mitigating the negative effects of cold stress is key to ensuring agricultural stability and food security.

Over the years, researchers have proposed various strategies to cope with cold stress, covering a range of methods from traditional agricultural techniques to modern biotechnology. These approaches include the breeding of cold-tolerant varieties, optimization of agricultural management practices, and the use of microbial resources, which has gained widespread attention in recent years. Traditional methods focus on external regulation, such as adjusting planting times, optimizing irrigation and fertilization strategies, and breeding adaptable varieties, to improve rice's ability to withstand cold environments (Ye et al., 2009). These methods have certain effects in practical production, but they often have limitations when dealing with extreme climate events, especially when addressing sudden cold events like cold waves and frosts in high-latitude regions.

In recent years, with a deeper understanding of plant-microbe interactions, the potential of microorganisms in enhancing rice cold tolerance has gradually emerged. Research has shown that certain microorganisms can effectively alleviate the negative effects of cold stress by regulating plant physiological metabolism,

enhancing antioxidant capacity, and activating cold-related genes (Balasjin et al., 2024; Shi et al., 2024). This biotechnological approach not only provides a new perspective for improving rice cold tolerance but also opens important avenues for achieving sustainable agricultural development.

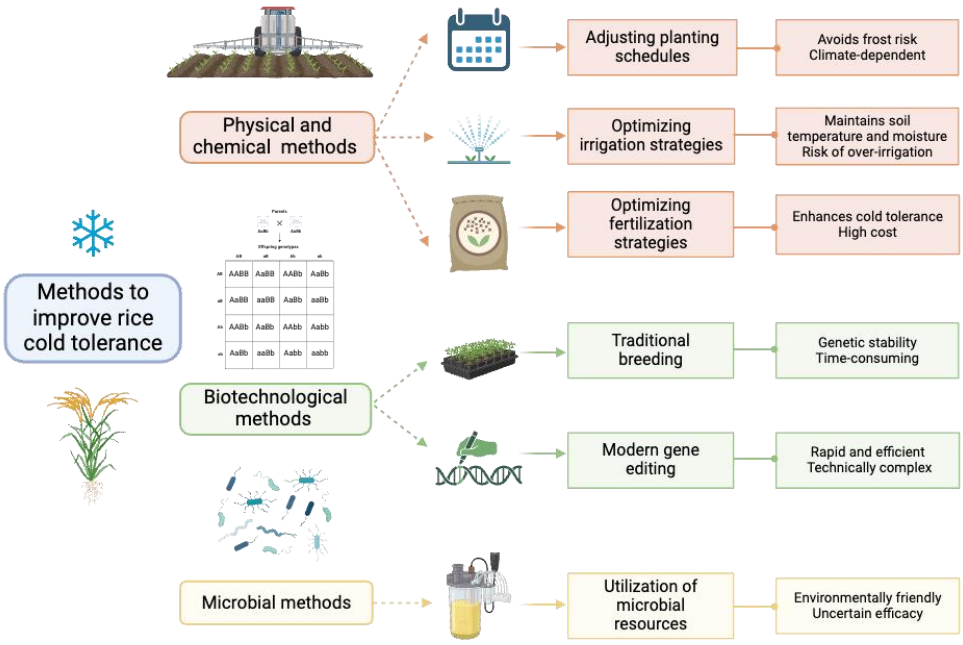

Figure 2.3 Methods to improve rice cold tolerance. Created with BioRender.com.

This section will systematically analyze various methods for improving rice cold tolerance, from traditional agricultural management measures to microbial methods, and further explore innovative strategies centered around seed endophytic microorganisms, discussing their specific roles and potential application value in alleviating cold stress.

2.2.1 Traditional methods for enhancing rice cold tolerance

Cold stress is one of the major abiotic stresses affecting rice growth and yield, and it is a key environmental factor limiting rice production efficiency in high-latitude and high-altitude regions. Traditional methods have played an important role in addressing cold stress, mainly including physical methods that regulate environmental conditions, chemical methods that control with chemical substances, and biotechnological methods based on breeding and genetic engineering. These methods have enhanced rice cold tolerance from different angles, providing diverse solutions to mitigate the adverse effects of low temperatures on rice. However, despite some success in practical application, these methods still have limitations in

terms of application scope and effectiveness duration.

2.2.1.1 Physical methods to improve rice cold tolerance

Physical methods regulate the growth environment of rice to reduce the direct impact of cold stress on rice. They are the most basic and widely applied strategies among traditional methods. In cold regions, adjusting the sowing time to avoid high-risk cold periods is a simple and effective approach (El-Refaee et al., 2024). However, climate uncertainty makes this method risky in the context of frequent extreme cold events. In addition, mulching techniques are another common physical method. For example, using plastic films or straw to cover the soil during cold seasons can significantly raise soil temperature, improving seed germination and seedling growth conditions (Iqbal et al., 2020). On the other hand, greenhouse cultivation involves artificially controlled temperatures to provide stable environmental conditions for rice growth. This method is significant in seed breeding and the production of high-value crops, but due to its high cost and technical requirements, it is difficult to apply to large-scale production.

2.2.1.2 Chemical methods to improve rice cold tolerance

Chemical methods regulate rice cold tolerance by externally applying chemical substances. Significant progress has been made in both research and application of these methods in recent years. These chemical substances include plant hormones, antifreeze agents, and nutrient regulators. Plant hormones play a crucial role in regulating plant stress tolerance. For instance, ABA enhances rice's cold tolerance by activating the expression of cold-related genes and stabilizing the cell membrane (Wang et al., 2022b). Additionally, seed treatments with salicylic acid (SA) can promote seed germination rate, seedling vigor, and seed quality under cold stress. They also enhance the activity of stress-related enzymes, such as peroxidase, catalase, and superoxide dismutase (S.R et al., 2023). However, the effectiveness of plant hormones is highly influenced by environmental conditions and application methods, and their practical effect in production is often less significant than under experimental conditions.

Furthermore, studies have shown that applying chitosan improves seedling cold tolerance by repairing photosynthetic damage, altering osmotic regulation, and reducing oxidative damage (Zhou et al., 2018). In addition, the rational application of nitrogen, phosphorus, and potassium fertilizers as nutrient regulators can improve the physiological status of rice and enhance root activity, indirectly increasing cold tolerance (Liu et al., 2019). However, the application of chemical methods is often limited by cost, environmental factors, and efficiency, requiring careful selection and optimization according to specific situations.

2.2.1.3 Biotechnological methods to improve rice cold tolerance

Biotechnological methods primarily improve rice cold tolerance through genetic improvement and breeding technologies at the genetic level. These methods include traditional breeding, the development of new varieties, and the rapidly advancing field of genetic engineering. Traditional breeding, which involves selecting and crossbreeding cold-tolerant varieties, remains the foundational approach for

enhancing rice cold tolerance (Akter et al., 2022; Cao et al., 2022). For instance, cold-tolerant rice varieties developed in regions vulnerable to cold stress exhibit strong cold tolerance and higher yields, and they have been widely applied in production (Cruz et al., 2013). However, traditional breeding is typically time-consuming and constrained by natural conditions, making it less effective in addressing extreme cold events brought about by climate change.

In recent years, whole-genome analysis/sequencing, mutation studies, and transgenic plant research have significantly accelerated the breeding process (Sanghera et al., 2011; Shakiba et al., 2017). For example, by identifying cold tolerance-related genes such as *CBF* and *DREB*, researchers can rapidly screen and breed varieties with enhanced cold tolerance (Wang et al., 2008; Xu et al., 2011). Gene editing technologies, such as CRISPR/Cas9, have provided powerful tools for genetic improvement in rice (Zeng et al., 2020; Najeeb et al., 2021). In recent years, studies of cold-tolerance genes and their epigenetic regulation have provided powerful tools for improving rice resilience. DNA methylation enables rice to "remember" prior cold exposure and pass this adaptive tolerance to progeny (Song et al., 2025). In maize, a high‐latitude variant of *COOL1* enhances germination and seedling vigor under low temperatures (Zeng et al., 2025). In rice, *COLD1* encodes a GPCR-like protein that boosts chilling tolerance by modulating calcium signaling (Ma et al., 2015). Together, these discoveries suggest that integrating targeted gene editing with epigenetic strategies can accelerate the development of highly cold‐tolerant crop varieties. However, the promotion of genetic engineering technologies still faces challenges, including policy restrictions, ethical concerns, and public acceptance issues.

In conclusion, traditional methods have made notable progress in enhancing rice cold tolerance. From the simplicity and practicality of physical methods, the efficient and direct chemical regulation approaches, to the precise and effective biotechnological techniques, these technologies offer diverse options to tackle cold stress. However, each method has its limitations. For example, physical methods are restricted by environmental conditions, chemical methods may be costly and pose environmental risks, and biotechnological methods require higher technical investment and policy support. Therefore, future research should build upon existing technologies, combining interdisciplinary collaboration and innovation to develop more sustainable and adaptable comprehensive solutions.

2.2.2 Microbial approaches to enhance rice cold tolerance

Microbial methods have gained widespread attention in recent years as an eco-friendly and sustainable agricultural strategy to combat cold stress and improve rice cold tolerance. This approach leverages beneficial microbes, either naturally occurring or artificially selected, to significantly enhance rice survival under cold conditions by improving plant growth environments and directly regulating physiological metabolism. Compared to traditional agricultural techniques, microbial methods not only reduce the use of chemical fertilizers and pesticides but also demonstrate advantages such as being green, efficient, and cost-effective.Rice and its interactions with microorganisms from both its inherent microbiome and the

external soil microbiome (Zhao et al., 2024b). The inherent rice microbiome includes microbial entities present in different rice tissues, such as pollen, seeds, straw, leaves, and roots. Complex differences can be observed in the microbial composition within a single tissue. For example, root-associated microbes can be divided into the endosphere (inside the roots), rhizoplane (on the root surface), and rhizosphere (the surrounding soil zone). Similarly, leaf microbes can be categorized into endophytic, epiphytic, and surface-attached microbial communities. Among these microbes, soil microbes and seed endophytic microbes are of particular interest due to their unique ecological niches and functional roles. Soil microbes not only influence the nutrient acquisition of rice roots but also regulate plant growth and tolerance through interactions with the plant roots. Furthermore, seed endophytic microbes, as the initial microbial source for rice, can colonize during the early stages of rice growth and have a profound impact on its subsequent growth, health, and stress tolerance. Therefore, in-depth research into the characteristics, functions, and interactions of these microbes with rice is of significant scientific importance and holds great potential for practical applications.

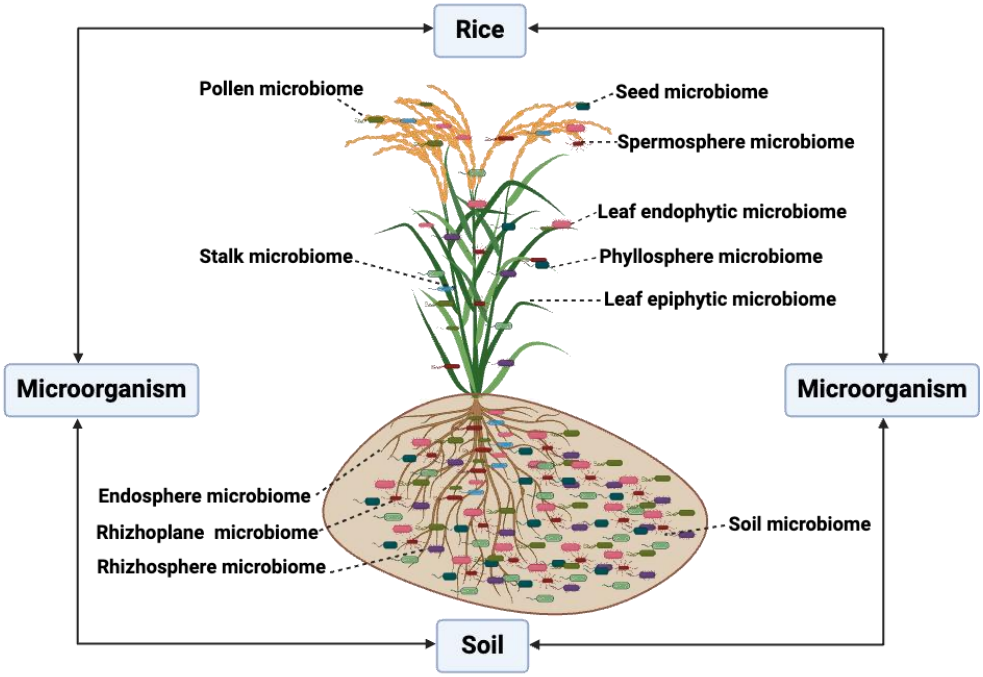

Figure 2.4 Origins of microorganisms interacting with rice (Zhao et al., 2024b).

2.2.2.1 Soil microbes in enhancing rice cold tolerance

The role of microbes in enhancing rice cold tolerance mainly involves mechanisms such as regulating plant hormone levels, secreting antifreeze proteins and antioxidant enzymes, enhancing nutrient absorption, and inducing cold-tolerant gene

expression (Shi et al., 2024). Soil microbes play a central role in improving the rice rhizosphere environment and boosting plant resilience to stress. These microbes help by optimizing nutrient cycling, secreting antifreeze substances, and activating plant defense mechanisms, which provide rice with effective adaptation to low temperatures.

Among these, plant growth-promoting bacteria (PGPB) have become a key focus due to their diverse functions. These bacteria promote root development and branching by secreting plant hormones like IAA), which enhances the plant's ability to absorb essential nutrients (Orozco-Mosqueda et al., 2023). Additionally, PGPB can secrete antifreeze proteins and antioxidant enzymes such as POD, SOD, and CAT, reducing oxidative damage to cell membranes caused by low temperatures. PGPB also increase nutrient availability by dissolving insoluble phosphates and fixing nitrogen.

Souza et al. isolated 9 strains of PGPB from the rhizosphere soil of cold-stressed rice fields and inoculated them into cold-stressed rice plants. Among these, two strains, *Kosakonia sp*. CIR2 and *Staphylococcus sp*. CSR1T2, conferred cold tolerance to the rice plants (de Souza et al., 2021). The inoculated rice plants exhibited higher survival rates, reached the reproductive stage approximately 25 days earlier, and showed improved fertility and yield parameters compared to the non-inoculated controls.

In another study, Tiwari et al. isolated a strain of *Bacillus amyloliquefaciens* (strain SN13) from soil, which conferred cold tolerance to rice by regulating osmotic protection and the expression of osmotic-related genes (Tiwari et al., 2017).

2.2.2.2 Seed endophytic microorganisms enhance cold tolerance in rice

Although microbial methods have shown great potential in improving rice cold tolerance, current research has primarily focused on the role of exogenous microorganisms, such as soil microorganisms and rhizosphere-promoting bacteria. In fact, endogenous microorganisms within plants, especially seed endophytes, play a critical role in enhancing cold tolerance during the early growth stages of the plant. In recent years, seed endophytic microorganisms have become a new hotspot in the study of improving crop resilience due to their unique physiological characteristics and mechanisms of action. Seed endophytic microorganisms naturally reside within plant seeds and can form a close symbiotic relationship with the host plant without causing disease, providing protection and support to the plant at various stages from germination to seedling growth (Mao et al., 2023). These microorganisms enhance cold tolerance by regulating plant physiological metabolism, increasing antioxidant capacity, and promoting nutrient absorption, thereby playing a key role in the early stages of the plant's life cycle and supporting plant growth and development under cold conditions (Zhang et al., 2021). Moreover, seed endophytes have the natural advantage of vertical transmission, allowing them to pass from the parent plant to the progeny seeds. This enables them to influence plant growth and development naturally, without the need for external inoculation, thereby ensuring stable intergenerational transmission and symbiosis that is unaffected by external environmental fluctuations (Shahzad et al., 2018; Wang and Zhang, 2023). The

stable microbial communities formed by seed endophytes not only provide nutritional support during seed germination but also significantly enhance seedling cold tolerance and stress tolerance. Exogenously inoculated microorganisms can also colonize the seeds and, after seed germination, can be detected in plant tissues, thereby affecting plant growth and development (Berg and Raaijmakers, 2018). Therefore, in-depth research into the role of rice seed endophytic microorganisms in enhancing cold tolerance will not only broaden the scope of plant stress-resilience biotechnology but also provide new ideas and technical support for developing novel green microbial agents in agricultural production.

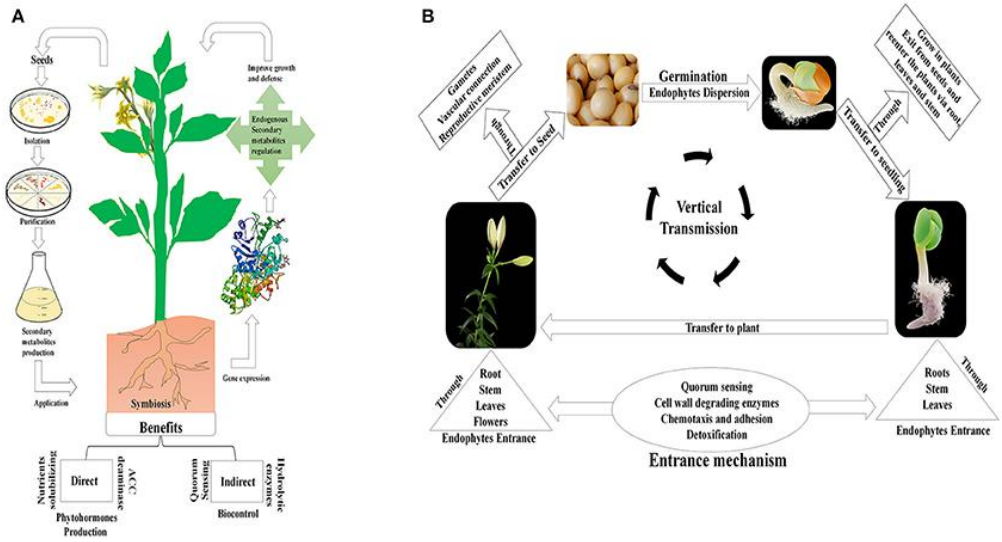

Figure 2.5 Conceptual view of mechanisms of action and vertical transmission of seed endophytic microbiota. (A) The schematic presentation shows the isolation of seed-borne endophytic microbes, and their application in promoting plant growth and stress tolerance. (B) A holistic view of the vertical transmission of seed-borne endophytes. This suggests that endophytes are found in seed embryos and grow into the emerging leaf upon germination; the endophytes then migrate into the stem and seed head of reproductive plants via various pathways (Shahzad et al., 2018).

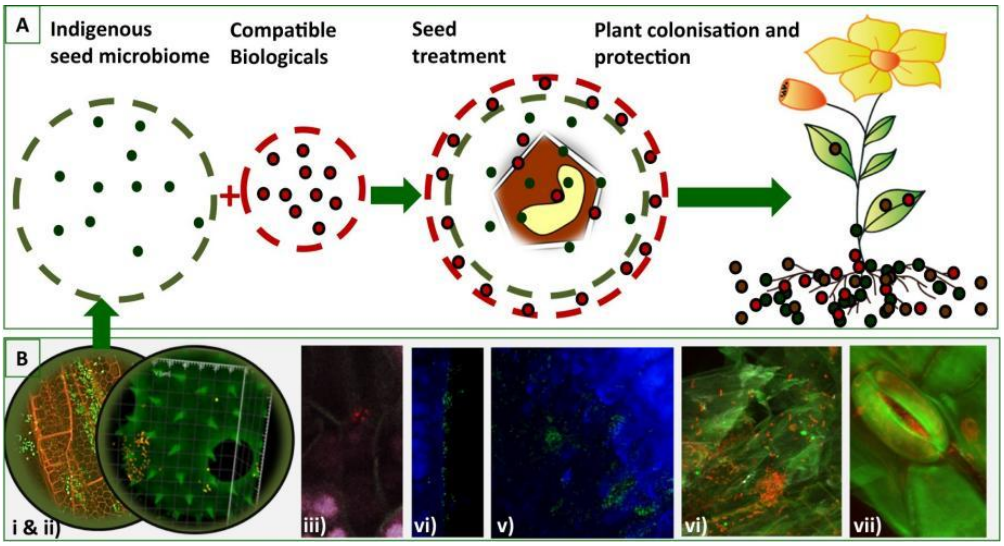

Figure 2.6 The seed microbiome. a showing the concept of compatible biologicals for crops and, b visualizing endophytes by in situ hybridization and confocal laser scanning microscopy (i) in the endosphere, (ii) on pollen, (iii) naturally occurring in seeds, (iv) after seed treatment within seed on the cotyledon, (v) after seed treatment within seed on the root hypocotyl embryo, (vi) in the rhizosphere and (vii) phyllosphere after seed treatment. Seeds were treated with Serratia plymuthica (Berg and Raaijmakers, 2018).

Rice seeds refer to the structures capable of developing into a new individual, including parts such as the embryo, seed coat, and endosperm. As the reproductive structure of the plant, rice seeds also serve as the carrier for its endophytic microbial communities, which play a crucial role in rice growth, development, and stress tolerance. The rice fruit develops from the mature ovary and contains the rice seed (Chen et al., 2024). Although rice seeds are closely related to rice fruit in botanical terms, and in the Poaceae family, the fruit and seed are typically the same structure, in studies of the endophytic microbiota of rice seeds, the seed is often the focus of biological research, particularly in relation to its microbial community (Zhou et al., 2020; Zhang et al., 2022). The microbial community within rice seeds plays an essential role in the plant's growth process. Studies have shown that specific endophytic bacteria accumulate in rice seeds during maturation, which promote colonization and growth during the rice seedling stage (Dutta et al., 2022). Furthermore, the relationship between rice seeds and their microbial communities continues to be explored. Research indicates that the composition of the microbial community within rice seeds undergoes dynamic changes in response to the growth stages of the rice plant and environmental conditions, which in turn affects rice growth and development (Nanfack et al., 2024).

Seed endophytic microorganisms improve rice cold tolerance primarily through the regulation of plant hormone levels, promotion of nutrient absorption, secretion of antifreeze proteins and antioxidants, and modulation of ethylene synthesis, among

other synergistic mechanisms. Firstly, seed endophytic microorganisms exhibit various plant growth-promoting traits, which in turn regulate plant hormone levels and promote nutrient absorption (Krishnamoorthy et al., 2020; Zhang et al., 2021). For example, Ionel isolated an endophytic bacterium from Cuban rice seeds that produces auxins, dissolves phosphate and potassium, and generates iron carriers. Inoculating rice with these strains resulted in increased height, root length, fresh weight, and dry weight of both the shoot and root systems (Hernández et al., 2023). Additionally, certain endophytic bacteria and fungi are capable of secreting antifreeze proteins (AFP) and antioxidant enzymes (such as SOD, POD, and CAT), which are crucial defense substances for plants in response to cold stress. These substances effectively reduce ice crystal damage to plant cell membranes, stabilize membrane structures, decrease mechanical damage caused by ice crystals, reduce the accumulation of ROS, and alleviate oxidative damage caused by cold stress (Juurakko et al., 2021). Furthermore, seed endophytic microorganisms can regulate ethylene synthesis by secreting 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Walitang et al., 2017). Ethylene plays a significant role in the plant's response to environmental stress and is an important signaling molecule produced when the plant is under stress. Excessive ethylene accumulation can inhibit plant growth and exacerbate stress. Seed endophytic microorganisms reduce ethylene synthesis, thereby alleviating the stress response and promoting seed germination and seedling growth under stress conditions.

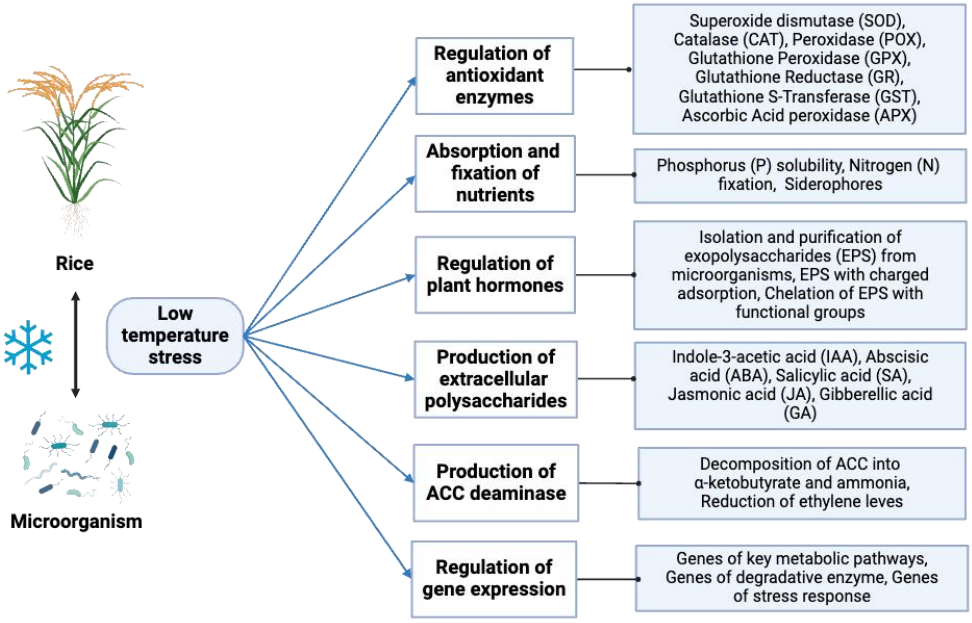

Figure 2.7 Mechanisms by which microorganisms enhance rice cold tolerance. Created with

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2.2.2.3 Microbial preparations and their application in rice

Microbial inoculants are products made from selected and cultivated beneficial microorganisms, which are applied in agricultural production through methods such as spraying, root irrigation, or seed coating to improve the soil microenvironment and enhance crop stress tolerance (Zhao et al., 2024b). In recent years, microbial inoculants containing phosphorus-solubilizing bacteria, nitrogen-fixing bacteria, and antifreeze microorganisms have been widely applied in rice cultivation. For example, phosphorus-solubilizing bacteria secrete organic acids to dissolve insoluble phosphate salts, significantly improving the phosphorus absorption efficiency in rice; nitrogen-fixing bacteria, on the other hand, fix atmospheric nitrogen, enhancing the nitrogen supply to rice and promoting its growth. Additionally, some composite microbial inoculants combine the advantages of PGPB and antifreeze microorganisms, significantly improving rice growth and yield under cold conditions through various synergistic mechanisms. Feng et al. used plant growth-promoting bacteria (*Agrobacterium rhizogenes* and *Bacillus subtilis*) as auxiliary bacteria to enhance the function of the cold-tolerant Indian pear-shaped fungus, which further increased rice cold tolerance (Shi et al., 2024).

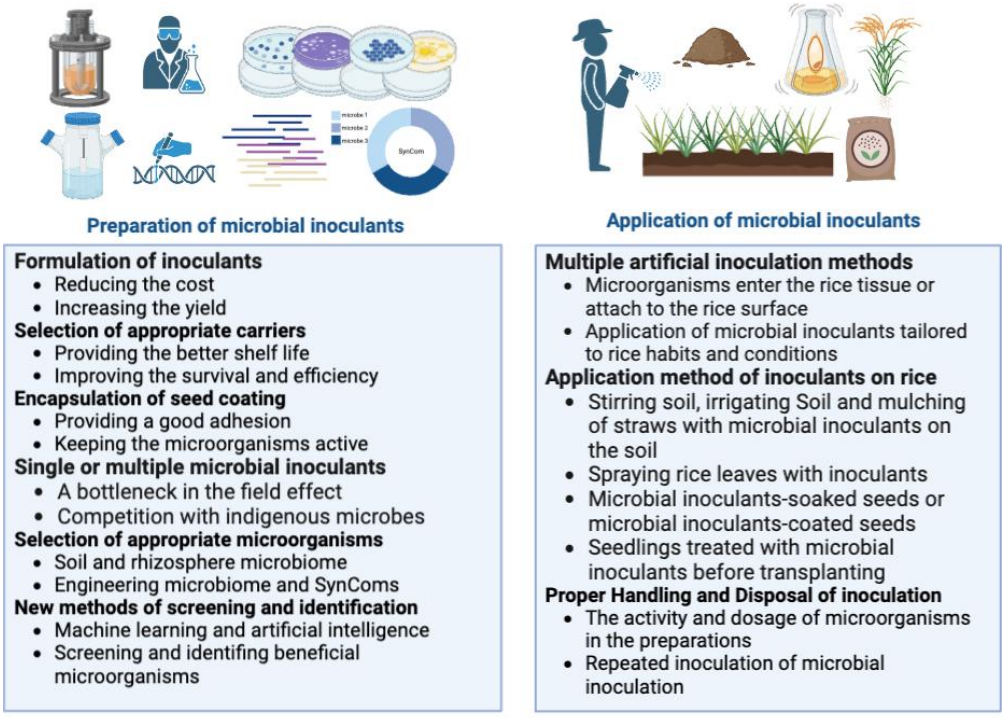

Figure 2.8 Application of microbial inoculants in rice cultivation (Zhao et al., 2024b).

With the deepening research into microbial functions and mechanisms, building symbiotic systems between plants and microorganisms has become a new research focus. By screening and combining microorganisms with different functions, and using microbiome engineering approaches to create synergistic microbial communities, sustainable rice production can be promoted (Misu et al., 2025). For instance, César developed a composite biofertilizer using six cold-tolerant bacteria isolated from wild rice roots. This biofertilizer promotes plant nutrient absorption while enhancing the plant's antioxidant capacity and hormone regulation, thereby improving the tolerance of rice (*Oryza sativa* L.) to cold stress (Valle Expósito et al., 2022). The construction of such composite microbial inoculants and symbiotic systems not only improves the survival rate and yield of rice in cold environments but also enhances the stability of agricultural ecosystems, promoting sustainable agricultural development.

The effectiveness of microbial inoculants in field applications is influenced by various factors, such as the type of microorganism, environmental conditions, and application methods (Khan et al., 2023a) For example, in cold, high-latitude regions, low temperatures may inhibit microbial activity, affecting the efficacy of the preparation. Therefore, future research should focus on optimizing the formulation and application strategies of microbial inoculants according to different regions and environmental conditions to maximize agricultural benefits. Microbial methods have shown great potential in enhancing rice cold tolerance. Future research should continue to explore the mechanisms of microbial action, develop more powerful microbial inoculants, and combine modern biotechnologies (such as gene editing and microbiomics) to further optimize microbial application strategies. This green and sustainable agricultural model will play an important role in addressing global climate change and ensuring food security, providing strong technical support for sustainable agricultural development.

2.3 Microbacteriaceae microorganisms

2.3.1 Classification and ecological distribution of Microbacteriaceae

Microbacteriaceae, a family of bacteria in the order Actinomycetales and class Actinobacteria, is a highly diverse and functionally versatile group. It includes several genera such as *Microbacterium*, *Agromyces*, *Leifsonia*, and *Curtobacterium* (Evtushenko and Takeuchi, 2006). These bacteria are characterized by high GC content and Gram-positive properties, predominantly exhibiting rod-shaped or coccoid morphologies. They are widely distributed in terrestrial, freshwater, and marine ecosystems, with a particularly prominent presence in plant endophytic communities, forming one of the core components of plant-associated bacteria (Vasilenko Oleg et al., 2018). High-throughput sequencing studies have shown that Microbacteriaceae microorganisms are widely present in plant seeds, roots, and leaf tissues, with their distribution and function varying depending on the host plant species, ecological environment, and stress conditions (Tarlachkov Sergey et al.,

2020).

Microbacteriaceae microorganisms have significant potential in promoting plant growth, enhancing crop stress tolerance, and contributing to ecosystem services. For example, in drought-resistant rice seeds, Microbacteriaceae has been confirmed as an important component of the endophytic bacterial community. These microorganisms regulate plant hormone levels and promote mineral nutrient absorption, enhancing seed germination rates and stress tolerance (Wang et al., 2021b). Furthermore, in both salt-sensitive and salt-tolerant rice varieties, Microbacteriaceae microorganisms demonstrate strong endophytic colonization ability and plant growth-promoting propertie (Walitang et al., 2017). These studies indicate that Microbacteriaceae not only play a critical role as key members of plant endophytic bacteria but also play an essential role in improving plant stress tolerance.

2.3.2 Interaction between Microbacterium testaceum and plants

Microbacterium testaceum is an important representative species of the Microbacteriaceae family. It is a Gram-positive, rod-shaped, yellow-orange bacterium that was first isolated from rice by Komagata K and Iizuka H. After further taxonomic revision, it was classified under the genus *Microbacterium* and named *Microbacterium testaceum* (Kazuo Komagata 1964; Yamada and Komagata, 1972; Collins et al., 1983; Takeuchi and Hatano, 1998). This bacterium can symbiotically associate with plants without causing diseases and exhibits a variety of beneficial biological properties. Studies have shown that *Microbacterium testaceum* is widely distributed in plant endophytic communities and often acts as an important member of plant-associated microbes, promoting plant growth and enhancing plant tolerance to both biotic and abiotic stresses through various mechanisms (Zinniel Denise et al., 2002; Zinniel et al., 2008).

Microbacterium testaceum promotes plant growth by secreting plant hormones. Studies have found that this bacterium can synthesize plant hormones such as IAA, which regulates plant growth and development. For example, a *Microbacterium testaceum* strain (Y411) isolated from the aerial roots of orchids has been shown to be an efficient IAA producer, significantly promoting root development and plant nutrient uptake (Yadav et al., 2022).

In terms of enhancing plant stress tolerance, *Microbacterium testaceum* exhibits remarkable functional advantages. Regarding disease resistance, secondary metabolites secreted by *Microbacterium testaceum* (such as Testacosides A-D) and N-acyl homoserine lactone (AHL) degrading enzymes (AHLase) effectively inhibit the growth of various plant pathogens, reducing the risk of pathogen infection in plants (Morohoshi et al., 2011; Quintana-Bulla et al., 2024). *Microbacterium testaceum* can reduce the use of chemical pesticides by inhibiting the growth of *Magnaporthe oryzae* (rice blast fungus) and *Phytophthora infestans* (potato late blight), thus decreasing the negative environmental impacts of agricultural production (Patel et al., 2022; Patel et al., 2023).

Microbacterium testaceum also exhibits quorum sensing inhibition capabilities. For instance, *Microbacterium testaceum* strains BAC1065 and BAC1093 isolated from bean plants can block pathogenic expression by degrading the signaling molecules of pathogens, thereby inhibiting their quorum sensing mechanisms (Lopes et al., 2015). Additionally, the AHL-degrading ability of *Microbacterium testaceum* has garnered widespread attention. A strain isolated from potato leaf surfaces, *Microbacterium testaceum* StLB037, can degrade AHL, effectively preventing plant pathogen infection (Wang et al., 2010). Furthermore, *Microbacterium testaceum* isolated from sugar beet demonstrates strong antimicrobial activity, highlighting its natural antagonistic potential against plant pathogens (Zachow et al., 2008). These studies suggest that *Microbacterium testaceum* plays a significant role in the biotic stress management of plants.

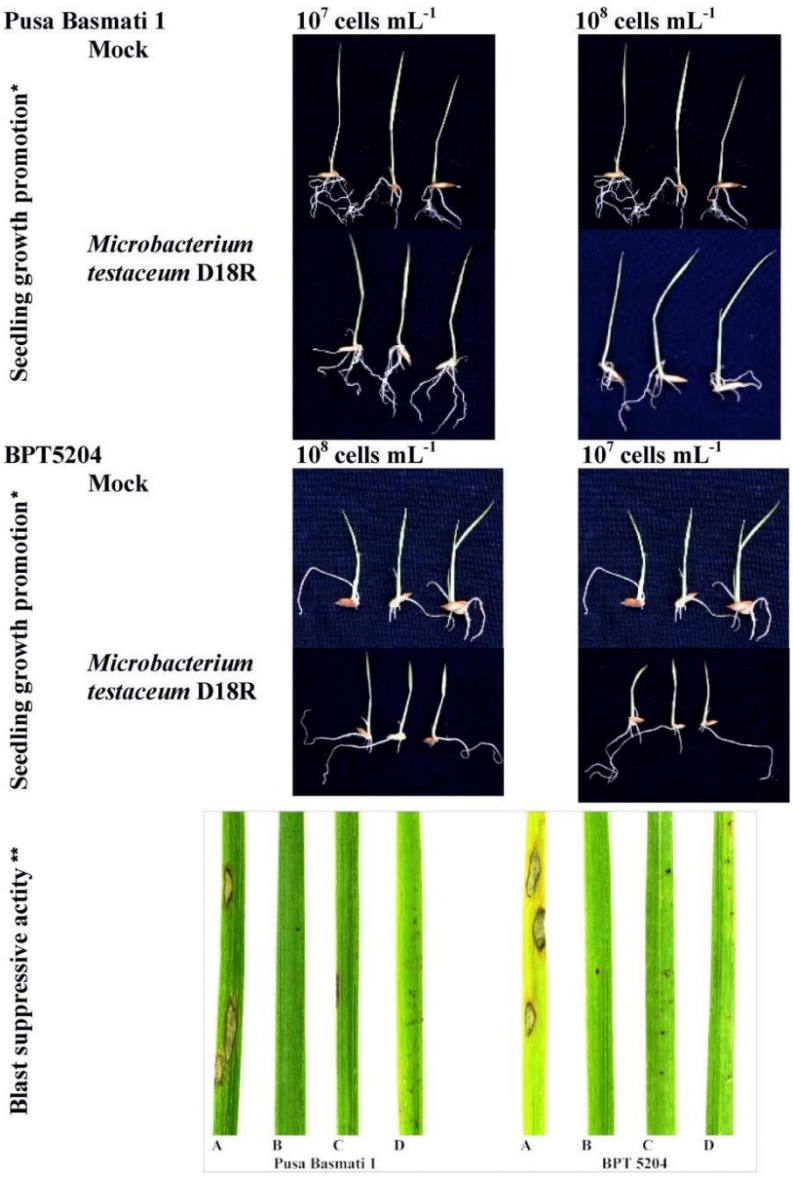

Figure 2.9 *Microbacterium testaceum* D18 induced growth promotion in rice seedlings, and blast disease suppressive activity. **M. testaceum* stimulated the growth of rice seedlings upon bacterization; ***M. testaceum* D18 triggered a hypersensitivity type of reaction instead of large lesions observed in mock; the hypersensitive reaction indicates over-expression of defense genes. The number of lesions and size of lesions were found reduced in bacterized plantlets; plant responses are scored as per Mackill and Bonman. (A) Mock. (B) *M. testaceum* D18 (108 CFU mL − 1). (C) *M. testaceum* D18 (107 CFU mL − 1). (D) D. Tricyclazole (Patel et al., 2022)

However, despite considerable research on the ability of *Microbacterium testaceum* to enhance plant tolerance to biotic stresses, there are still relatively few reports on its role in improving plant tolerance under abiotic stress conditions such as low temperature, high salinity, and drought. Its functional mechanisms in these areas remain underexplored. *Microbacterium testaceum* has promising potential for agricultural applications, and its remarkable abilities to promote plant growth and enhance stress tolerance make it a valuable resource for the development of green agricultural microbial formulations. Future studies should further integrate genomics, transcriptomics, and metabolomics technologies to comprehensively elucidate the functional mechanisms of *Microbacterium testaceum*, laying the theoretical foundation for the development of multifunctional microbial agents.

2.4 Phosphate-solubilizing microorganisms

Phosphorus (P) is one of the essential nutrients for plant growth and development. It plays a central role in critical physiological processes such as photosynthesis, energy metabolism, nucleic acid synthesis, and cell division, and influences seed germination, root growth, as well as the formation of flowers and seeds (Malhotra et al., 2018). In addition, phosphorus utilization efficiency is also related to a plant's tolerance to environmental stress (Bechtaoui et al., 2021; Khan et al., 2023b). However, despite the abundant total phosphorus content in soils, the available phosphorus that can be directly absorbed and utilized by plants is very limited. This is because most phosphorus exists in insoluble mineral forms (such as calcium phosphate, iron phosphate, and aluminum phosphate) or is fixed in the soil in the form of complex organic phosphorus compounds, making it difficult for plants to directly absorb and utilize it (Bhattacharya, 2019; Ibrahim et al., 2022). To meet the phosphorus demand of crops, traditional agricultural production often relies on large-scale application of chemical phosphorus fertilizers, but this approach not only increases agricultural costs but also causes significant environmental pollution, such as soil compaction and water eutrophication. How to efficiently and environmentally utilize phosphorus resources has become a key challenge for sustainable agricultural development.

In this context, phosphate-solubilizing microorganisms (PSMs) have gained attention as important tools for improving soil phosphorus utilization. These microorganisms can convert insoluble phosphorus into forms that are accessible for plant uptake through various mechanisms (Cheng et al., 2023). Studies have shown that PSMs not only effectively promote the absorption of phosphorus by plants and play a crucial role in plant growth and reproduction, but they also improve the soil microecological environment, reduce the reliance on chemical phosphorus fertilizers, and lessen the negative environmental impacts of agricultural production. (Sharma et al., 2013b; Iftikhar et al., 2024). Especially in cold regions, where low temperatures limit phosphorus conversion and plant uptake, cold-tolerant PSMs have shown great potential in improving crop yield and tolerance to stresses (Rizvi et al., 2021). Therefore, systematically studying the mechanisms of PSMs, screening excellent functional strains, developing efficient microbial formulations, and exploring their

application in different agricultural ecosystems are of great significance for achieving green agriculture and sustainable development (Kalayu, 2019; García-Berumen et al., 2025).

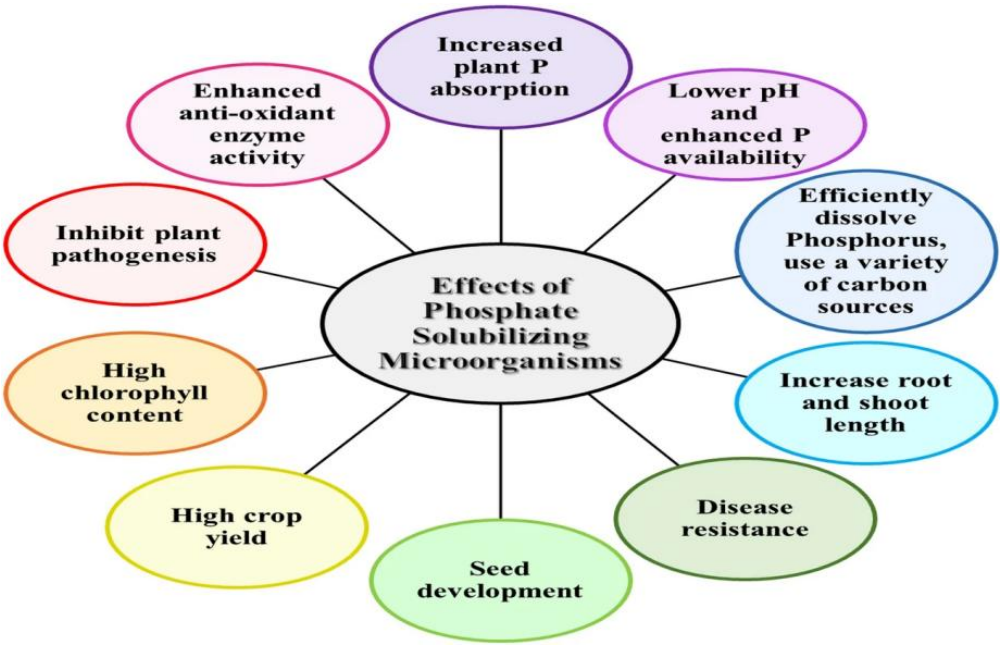

Figure 2.10 Role of PSMs in plant growth and propagation, increasing root length, high-yielding crops, seed development, etc (Iftikhar et al., 2024).

2.4.1 Mechanisms of Phosphate-solubilizing microorganisms

Phosphate-solubilizing microorganisms (PSMs) are a group of microorganisms capable of converting insoluble phosphate compounds in the soil into soluble phosphorus forms that plants can absorb and utilize. These microorganisms promote the release and transformation of phosphorus in the soil through various mechanisms, enhancing the efficiency of phosphorus absorption and utilization by plants (Pang et al., 2024a).

Firstly, organic acid secretion is one of the main mechanisms by which PSMs promote phosphate solubilization. PSMs can secrete various organic acids such as citric acid, oxalic acid, lactic acid, and acetic acid. These organic acids lower the pH of the rhizosphere soil, facilitating the dissolution of phosphate minerals (e.g., calcium phosphate, iron phosphate, and aluminum phosphate) (Rawat et al., 2021). Furthermore, these organic acids can form chelates with metal cations (such as Ca^{2+}, Fe^{3+}, and Al^{3+}) in the soil, disrupting the crystal structure of minerals and releasing the fixed phosphorus. This acidification and chelation process is an important pathway for PSMs to enhance the bioavailability of phosphorus.

Secondly, enzymatic hydrolysis plays a crucial role in the utilization of organic phosphorus by PSMs (Tian et al., 2021). PSMs secrete various phosphatases (such as acid phosphatase, alkaline phosphatase, and phytase), which convert complex organic phosphorus compounds in the soil into inorganic phosphorus forms that plants can directly absorb (Ughamba et al., 2025). The action of phosphatases is particularly significant in soils rich in organic matter. Phytase, for example, hydrolyzes phytic acid salts in plant residues, releasing phosphorus for plant utilization, a process that is especially important for improving phosphorus utilization efficiency (Bhattacharya, 2019).

Proton pump activity is also an important mechanism by which PSMs promote phosphate solubilization. Certain PSMs activate proton pumps to expel protons (H^+) from their cells, significantly lowering the pH of the rhizosphere soil (Silva et al., 2023). The lowered pH directly promotes the dissolution of phosphate minerals, releasing phosphorus in soluble forms for plant absorption.

Additionally, microbial metabolic products also contribute to promoting phosphorus release through chelation. Certain PSMs secrete iron carriers (siderophores) and exopolysaccharide (EPS), which form stable complexes with metal cations in phosphate minerals, effectively disrupting the mineral structure and promoting phosphorus release (Sharma et al., 2013b; Silva et al., 2023; Pang et al., 2024a). The production of these substances not only enhances the phosphate solubilizing ability of PSMs but may also impact the dynamic balance of other microbial communities in the soil, further optimizing the plant's growth environment.

Through various synergistic mechanisms, including organic acid secretion, enzymatic hydrolysis, proton pump activation, and chelation, PSMs effectively promote the dissolution and transformation of phosphorus in the soil. These mechanisms are of great significance for improving the phosphorus supply in the soil, enhancing plant phosphorus absorption efficiency, and reducing dependence on chemical phosphorus fertilizers. They provide critical support for achieving green agriculture and sustainable development.

2.4.2 Cold-tolerant Phosphate-solubilizing microorganisms

In cold or high-altitude regions, cold environments significantly inhibit the metabolic activity of soil microorganisms and nutrient cycling, especially the bioavailability of phosphorus, which decreases sharply. In such environments, plants have lower phosphorus absorption efficiency, which in turn affects the normal growth and yield of crops. Therefore, exploring microbial resources that can effectively function in cold environments has become crucial for improving agricultural productivity in cold regions. cold-tolerant phosphate-solubilizing microorganisms are a type of functional microorganisms that can maintain efficient phosphorus dissolution and promote plant growth in cold environments. These microorganisms not only survive and proliferate in cold conditions but also promote the dissolution of insoluble phosphorus in the soil and facilitate plant phosphorus absorption through a series of metabolic activities (Rizvi et al., 2021).

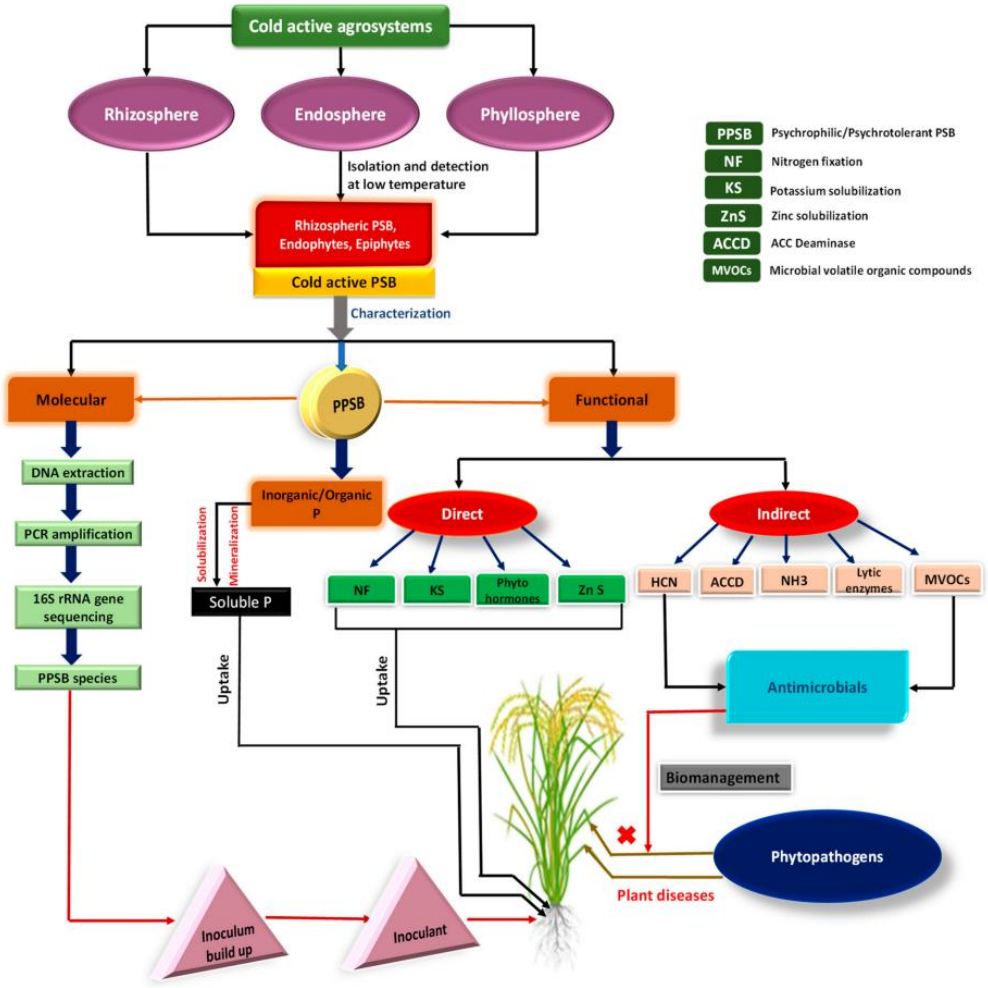

Figure 2.11 Cold-active phosphate biofertilizers: isolation, characterization, P solubilization, and plant growth promotion in the cold environment (Rizvi et al., 2021).

Cold-tolerant phosphate-solubilizing microorganisms promote growth not only by improving plant phosphorus absorption but also by enhancing plant stress tolerance. For example, cold-tolerant phosphorus-solubilizing *Pseudomonas* strains enhance wheat growth and yield by improving soil phosphorus nutrition (Dasila et al., 2023). cold phosphate and potassium-solubilizing bacteria have growth-promoting effects on soybeans in cold regions (Yan et al., 2025). Phosphate-solubilizing *Pseudomonas* strains isolated from high-altitude soils in the Himalayas can promote *Arabidopsis* growth under low temperatures (Adhikari et al., 2021). The inoculation of the psychrotolerant phosphate-solubilizing bacterium *Serratia nematodiphila* EU-PW75 alleviates cold stress and promotes growth in barley (*Hordeum vulgare* L.) (Kour

and Yadav, 2023). Research by Nasim shows that phosphate-solubilizing PGPB *Pseudomonas mosselii* improve cold tolerance in both indica and japonica rice varieties by increasing antioxidants such as reduced glutathione and proline (Balasjin et al., 2023). These microorganisms can regulate the plant's hormone balance, promote the synthesis of IAA, and inhibit ethylene accumulation through ACC deaminase, effectively alleviating growth inhibition under cold conditions. Furthermore, cold-tolerant phosphate-solubilizing microorganisms can enhance the activity of the plant's antioxidant system (such as increasing the activities of SOD, POD, and CAT, reducing oxidative damage caused by cold stress and further improving plant cold tolerance and stress tolerance.

Although cold-tolerant phosphate-solubilizing microorganisms hold great promise for agricultural production in cold regions, research into their mechanisms of action is still insufficient. Specifically, the molecular mechanisms by which they promote plant growth and improve cold tolerance have not been fully elucidated. Therefore, further in-depth studies on the functional mechanisms of cold-tolerant phosphate-solubilizing microorganisms will provide theoretical support and practical guidance for their application in agriculture in cold regions.

2.5 Conclusion

Cold stress is a major abiotic factor that significantly limits rice growth and productivity, particularly in high-latitude and high-altitude regions. Throughout the rice growth cycle—from germination to maturity—cold stress adversely affects physiological metabolism, photosynthetic efficiency, root development, and ultimately, grain yield and quality. With the increasing frequency of extreme weather events due to global climate change, understanding the impact of low temperatures on rice and developing effective strategies to enhance cold tolerance have become critical for ensuring agricultural stability and food security.

Conventional methods to improve rice cold tolerance, including breeding cold-tolerant varieties, optimizing agronomic practices, and applying chemical treatments, have achieved varying degrees of success. However, these approaches often face limitations in terms of sustainability, environmental impact, and adaptability to rapidly changing climatic conditions. Recent advancements in microbial technology have unveiled the potential of leveraging beneficial microorganisms to enhance rice cold tolerance.

Microbacteriaceae, particularly *Microbacterium testaceum*, play a crucial role in promoting rice growth and resilience under cold stress. These endophytic bacteria facilitate plant development by synthesizing phytohormones like IAA, enhancing nutrient uptake, and modulating antioxidant systems to mitigate oxidative damage. *M. testaceum* has demonstrated significant potential in both biotic and abiotic stress management, highlighting its promise as a bio-inoculant in sustainable agriculture.

PSMs represent another vital group contributing to rice cold tolerance. By converting insoluble soil phosphorus into bioavailable forms through organic acid secretion, enzymatic hydrolysis, proton extrusion, and chelation, PSMs improve

phosphorus uptake, which is critical for plant growth and stress tolerance. In cold environments, where phosphorus bioavailability is naturally reduced, cold-tolerant PSMs have shown to not only enhance nutrient acquisition but also bolster rice's physiological and biochemical responses to cold stress.

Seed endophytic microorganisms further contribute to rice cold tolerance by establishing stable, inheritable microbial communities that support plant growth from germination through maturity. These microbes regulate plant hormone levels, secrete antifreeze proteins and antioxidants, and modulate ethylene synthesis, collectively enhancing rice's resilience to cold stress.

The integration of these microbial strategies with traditional and biotechnological approaches offers a comprehensive framework for improving rice cold tolerance. Future research should focus on unraveling the molecular mechanisms underlying microbe-plant interactions, optimizing microbial consortia formulations, and applying multi-omics technologies to identify novel microbial functions. Such efforts will pave the way for the development of effective, eco-friendly microbial inoculants that not only enhance rice cold tolerance but also contribute to sustainable agricultural practices.

In conclusion, understanding the multifaceted impact of low temperatures on rice and harnessing the potential of beneficial microorganisms provides a promising avenue for mitigating cold stress. These insights will be instrumental in addressing the challenges posed by global climate change, ensuring stable rice production, and advancing sustainable agricultural development.

Chapter 3

Growth performance and endophytic microbial community characteristics of cold-tolerant and cold-sensitive rice varieties

This chapter shifts focus from the literature review to experimental research. It evaluates the cold tolerance and growth performance of two rice varieties—JG117 (cold-tolerant) and CB9 (cold-sensitive)—under both normal and cold conditions. The diversity and structural characteristics of the endophytic microbiomes in the seeds and seedlings of these two varieties are analyzed. The results in this chapter provide the basis for the subsequent isolation and functional characterization of endophytic microorganisms from rice seeds in Chapter 4.

This chapter is adapted from the following published research article:

Zhao J, Liu X, Hou L, Xu G, Guan F, Zhang W, Luo H, Wu N, Yao B, Zhang C, Delaplace P, Tian J. The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa* L.). Microbiological Research **2024**;289:127908. https://doi.org/10.1016/j.micres.2024.127908

3.1 Introduction

Rice (*Oryza sativa* L.) is a major staple food crop for half of the global population, playing a crucial role in food security and agricultural economics (Fukagawa and Ziska, 2019a). However, as a typical warm-season crop, rice is highly sensitive to cold stress, particularly during seed germination, seedling growth, and reproductive development stages (Walitang et al., 2017; Wang et al., 2021c). Cold stress can lead to reduced germination rates, stunted seedling growth, delayed flowering, insufficient grain filling, and ultimately a significant decrease in yield. This is especially true in temperate and high-latitude regions, where seasonal temperature fluctuations are pronounced, and cold stress has become a major environmental factor limiting both rice yield and quality. Therefore, improving the cold tolerance of rice has become a key goal in global rice production and breeding. Traditional cold tolerance improvement strategies mainly rely on gene screening and hybrid breeding, while modern cold tolerance strategies have developed biotechnologies such as gene editing. Although these methods have improved rice cold tolerance to some extent, there are still numerous limitations, such as restricted genetic diversity, long breeding cycles, technical complexity, and high costs (Sanghera et al., 2011; Villalobos-López et al., 2022). Consequently, exploring new and sustainable strategies for cold tolerance has become a critical direction in current rice research.

In recent years, an increasing number of studies have shifted focus to the interaction between plants and microorganisms, exploring the potential of utilizing endophytic microbes to enhance plant cold tolerance. Endophytic bacteria are microorganisms that reside within plant tissues without causing harm and have been proven to play an important role in promoting plant growth and enhancing stress tolerance. These endophytes can regulate plant hormone levels, improve nutrient absorption, and mitigate oxidative stress damage by activating the plant's antioxidant systems (Afzal et al., 2019). The interaction between plants and microorganisms is a crucial biological mechanism for plants to maintain growth and enhance productivity under adverse environmental conditions. Endophytic microorganisms not only participate in plant growth regulation but also help plants resist cold stress through multi-level physiological regulatory mechanisms (Ameen et al., 2024).

The symbiotic relationship between plants and microorganisms is a vital biological mechanism for plants to adapt to environmental stress, maintain growth, and increase productivity (Zhao et al., 2024a). Recent research has revealed that the plant genotype plays a decisive role in the composition and diversity of the endophytic microbiome (Zhang et al., 2023). Studies have shown that the endophytic microbiome in rice plays a significant role in plant stress tolerance. There are notable differences in the composition and diversity of the endophytic microbiome in rice varieties with different genotypes and stress tolerance (Balasjin et al., 2022). For example, research indicates that salt-tolerant rice varieties have a different diversity and community structure of endophytic bacteria in their seeds compared to salt-sensitive varieties, suggesting that endophytic bacteria may contribute to salt tolerance (Walitang et al., 2018). Similarly, studies have shown that plants that are tolerant to heavy metals possess endophytic populations with

high tolerance to heavy metals (Chu et al., 2021). Different genotypes of plant varieties form specific chemical niches via root exudates, which provide varied nutrients and signals to selectively attract and enrich specific microorganisms. This process regulates the recruitment and enrichment of rhizosphere and endophytic microbes. Enriched microbes form a unique microbial community structure that, through metabolic cooperation, creates a stable micro-ecosystem, thereby influencing the plant's ability to adapt to environmental stress. For instance, rice varieties with different genetic backgrounds show significant differences in cadmium concentrations in their roots, which also affects the specific bacterial communities associated with these plants (Chu et al., 2021). Furthermore, rice varieties that have long adapted to specific ecological environments may have formed stable co-evolutionary relationships with their symbiotic microorganisms (Wu et al., 2022). Domestication also impacts the evolution of the rice rhizosphere microbiome. For example, cultivated rice rhizospheres are more sensitive to rice blast disease than wild rice (Shi et al., 2018). In this process, the rice blast fungus produces oxalic acid and phenylacetic acid in the roots of the host plant, which nourish and activate specific rhizosphere microorganisms. Endophytic microorganisms, through their interaction with the plant genotype, jointly regulate the plant's stress response mechanisms.

Among functional endophytic microorganisms, the Microbacteriaceae family has attracted attention due to its potential in promoting plant growth and enhancing stress tolerance. Some strains from this family exhibit cold tolerance, such as *Cryobacterium mesophilum*, a psychrophilic strain isolated from glacier ice, which belongs to the Microbacteriaceae family (Liu et al., 2020). Other representatives, including species from the genus *Subtercol*a, have been found in cold environments, such as glacier ice, Arctic groundwater, and Antarctic sediments (Liu et al., 2020). In addition to strains isolated from cold environments, related microorganisms from the genus *Subtercola* have also been obtained from plants. *Subtercola endophyticus* sp. nov., a cold-tolerant bacterium AK-R2A1-2T isolated from Korean fir, is one such example (Jiang et al., 2022). Strain AK-R2A1-2T not only grows at 4℃ but also significantly improves the growth of rice seedlings under cold stress. Microbacteriaceae microorganisms, in addition to their ability to resist abiotic stress, can also cope with biotic stress. For instance, in the endophytic bacterial community of rice, the genus *Microbacterium* from the Microbacteriaceae family is a small, irregular, short, and slender rod-shaped Gram-positive bacterium. *Microbacterium* is a promising agricultural inoculant with biostimulatory activity and antimicrobial effects against pathogens. Epiphytic *Microbacterium testaceum* induces a defensive response on the leaf surface of the rice variety Pusa Basmati-1, thereby inhibiting rice blast disease (Sahu et al., 2021). Endophytic *Microbacterium testaceum* in rice can also confer immunity against rice blast disease (Patel et al., 2022). However, reports on the involvement of related Microbacteriaceae strains in rice cold tolerance are still lacking, and their functional characteristics and impact on rice need further exploration.

The phenotypic expression of rice is not only dependent on its genotype but also closely related to the composition and function of its endophytic microbiome.

Investigating the interaction between rice genotype and endophytic microorganisms, especially the role of Microbacteriaceae species in rice cold tolerance, will not only help reveal the plant's cold tolerance mechanisms but also provide new ideas and strategies for developing microbial inoculants, green agriculture, and sustainable food production. This study will evaluate the growth performance of two rice varieties with different cold tolerances under normal and cold stress conditions, and analyze the diversity and composition of their seed and seedling endophytic bacterial communities. Through the application of high-throughput sequencing technology and physiological growth parameter measurements, this study aims to uncover the potential role of endophytic bacteria, particularly Microbacteriaceae members, in enhancing rice cold tolerance. It provides innovative ideas for using microbial inoculation strategies to improve rice tolerance to cold stress.

3.2 Materials and methods

3.2.1 Cold tolerance assessment of two rice varieties

Plant materials and growth conditions: This study utilized two japonica rice varieties, JG117 and CB9, provided by the Rice Research Institute of the Jilin Academy of Agricultural Sciences, China. The seeds were surface-sterilized by soaking and shaking in 75% ethanol for 10 minutes, followed by five washes with sterile distilled water. They were then soaked and shaken in 10% sodium hypochlorite for 10 minutes and washed three times with sterile distilled water. After surface sterilization, the seeds were soaked in sterile distilled water at 30℃ for three days, germinated for one day in Petri dishes lined with moist filter paper, and planted in glass tubes containing 1/2 sterile MS nutrient solution supplemented with 0.4% agar. Each tube held five seeds, and each group contained five replicates. The plants were grown at 26℃ under a 14-hour light/10-hour dark photoperiod for seven days. Subsequently, they were subjected to cold stress at 4℃ under the same photoperiod for seven days, followed by recovery at 26℃ under the same photoperiod for seven days.

Growth and cold tolerance assessment: After treatment, the survival rate, root length, shoot length, and fresh weight of rice seedlings were measured. Root and shoot lengths were determined using a ruler, and fresh weight was measured using a precision balance. The survival rate was calculated using the following formula:

Survival Rate (%) = (Number of Surviving Seedlings / Total Number of Seedlings) × 100%

3.2.2 Microbial diversity analysis of rice seeds and seedlings

Sample preparation: For analysis of seed-associated endophytic microbes, 2 g of surface-sterilized seeds (JG117 and CB9) were ground into fine powder using sterile mortar and pestle. For seedling-associated endophytic microbes, seedlings of JG117 and CB9 were cultured in glass tubes containing 1/2 sterile MS nutrient solution and 0.4% agar under a 14-hour light/10-hour dark photoperiod at 26℃ for seven days. Samples were collected under normal conditions after seven days of cultivation and

after seven days of cold stress treatment at 4℃. Each group contained five replicates. Surface-sterilized rice seedlings were homogenized using sterile mortar and pestle, and the homogenate was used for endophytic microbial analysis. Genomic DNA was extracted from the ground seed and seedling samples using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., USA). The quality and concentration of DNA were assessed using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Inc., USA).

Microbial amplicon sequencing and analysis: The V3-V4 region of the 16S rRNA gene was amplified from the extracted DNA samples using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Each primer was tagged with a unique 8 bp barcode sequence to distinguish different samples. PCR amplification was performed using an ABI 9700 PCR system (Applied Biosystems, Inc., USA). The PCR products were purified using the Agencourt AMPure XP kit (Beckman Coulter, Inc., USA) and evaluated for concentration and fragment size using a Caliper LabChip GX Touch HT system (PerkinElmer, Inc., USA). Sequencing libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs, USA), and library quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). Paired-end sequencing (PE250) was performed on the Illumina MiSeq platform (Illumina, Inc., USA) by Novogene Bioinformatics Technology Co., Ltd. Raw sequencing data were processed using FastQC (Babraham Bioinformatics, UK) and trimmed using Trimmomatic (USA). High-quality reads were merged using Pear (v0.9.6), and chimeric sequences were removed using the Vsearch (v2.7.1) uchime algorithm. Operational taxonomic units (OTUs) were clustered using the Vsearch uparse algorithm with a similarity threshold of 97%. Taxonomic classification of OTUs was performed using BLAST against the Silva138 database with an e-value threshold of 1e-5.

3.2.3 Statistical analysis

All experimental data were collected from five independent replicates and expressed as mean ± standard error (SE). Comparisons between two groups were conducted using independent sample t-tests. All statistical analyses were performed using IBM SPSS Statistics 20 software (IBM Corp., USA). Graphs were created using Origin 2021 Pro software (OriginLab Corporation, USA). A p-value of less than 0.05 was considered statistically significant.

3.3 Results and discussion

3.3.1 Growth performance of the rice plants under normal conditions

We conducted a preliminary assessment of cold tolerance in two rice varieties, JG117 and CB9, during the seedling stage. The rice varieties JG117 (parental sources: Changbai 15 (♀), Changbai 16 (♂)) and CB9 (parental sources: Jijing 60 (♀), Dongbei 125 (♂)) were selected for this study because they represent different

genetic backgrounds with significant differences in cold tolerance. In the agricultural practices of Northeast China, JG117 is a cold-resistant variety, while CB9 is a variety with relatively poor cold tolerance. These characteristics make them ideal candidates for comparing cold tolerance during the seedling stage of rice.

The seedlings were cultivated in glass tubes containing half-strength Murashige and Skoog (1/2 MS) nutrient solution supplemented with 0.4% agar. After 7 days of growth under normal temperature conditions (26℃, RT), survival rate, root length, shoot height, and fresh weight were measured. Both varieties exhibited normal growth under control conditions (Figures 3.1A and B), with a survival rate of 100% (Figure 3.1C). The root lengths of JG117 and CB9 were 4.70 ± 0.73 cm and 4.68 ± 0.54 cm, respectively, showing no significant difference (Figure 3.1D). However, the shoot height of JG117 (22.14 ± 1.57 cm) was significantly greater than that of CB9 (17.55 ± 0.90 cm) (Figure 3.1E). On the other hand, the fresh weight of JG117 (0.59 ± 0.04 g) was lower than that of CB9 (0.76 ± 0.03 g) (Figure 3.1F).

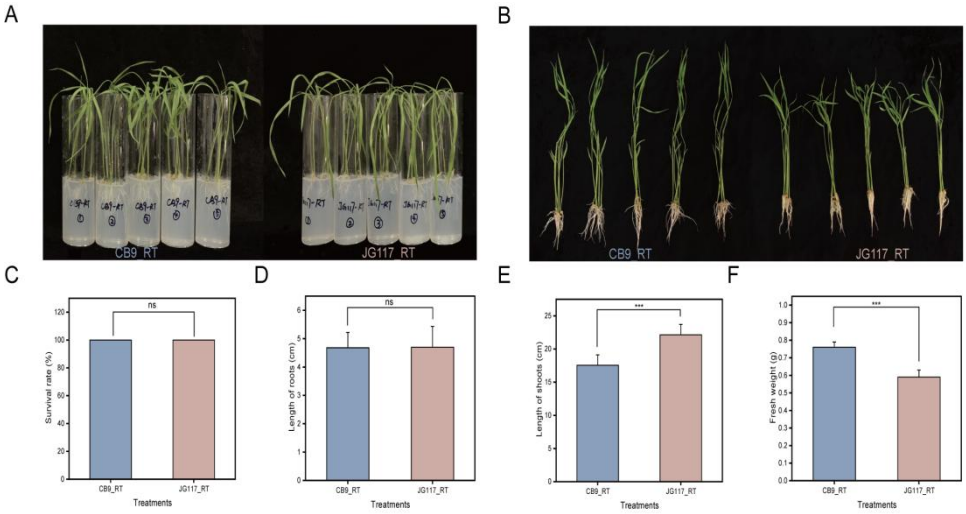

Figure 3.1 Growth performance of the rice varieties JG117 and CB9 under normal temperature conditions. (A) (B) Representative images of JG117 and CB9 seedlings grown for 7 days under normal temperature conditions (26℃, RT). (C) Survival rates of JG117 and CB9 seedlings after treatment at normal temperature (RT). (D) Root length of JG117 and CB9 seedlings after treatment at normal temperature (RT). (E) Shoot length of JG117 and CB9 seedlings after treatment at normal temperature (RT). (F) Fresh weight of JG117 and CB9 seedlings after treatment at normal temperature (RT). Error bars represent data from five independent biological replicates. ns indicates no significant difference, and *** indicates $p < 0.05$.

These results indicate that both rice varieties, JG117 and CB9, can grow normally under standard temperature conditions. While root growth showed no significant difference due to the short evaluation period, shoot growth differed significantly, with JG117 exhibiting longer shoots compared to CB9. Additionally, the lower fresh

weight of JG117 compared to CB9 may be attributed to differences in shoot morphology between the varieties; JG117 has slender shoots, whereas CB9 has shorter and thicker shoots, leading to differing biomass accumulation.

3.3.2 Growth performance of two rice varieties (JG117 and CB9) under cold conditions

The growth performance of the two rice varieties was further evaluated under cold conditions. Following 7 days of growth at normal temperature, 7 days of cold stress, and 7 days of recovery at normal temperature, the two varieties displayed different growth statuses. After recovery from cold stress, JG117 maintained normal growth with green leaves, whereas CB9 exhibited wilting and death after cold stress treatment (Figures 3.2A and B). Further measurements revealed a significant difference in survival rate, with JG117 achieving 96 ± 8.94% survival compared to only 8 ± 10.95% for CB9 (Figure 3.2C). Measurements of the aerial and root portions of the plants showed that the shoot and root lengths of JG117 (21.64 ± 2.43 cm and 5.06 ± 1.13 cm, respectively) were significantly greater than those of CB9 (17.73 ± 1.00 cm and 3.80 ± 0.64 cm, respectively) (Figures 3.2D and E). Additionally, JG117 had a higher fresh weight (0.67 ± 0.04 g) than CB9 (0.62 ± 0.05 g) (Figure 3.2F).

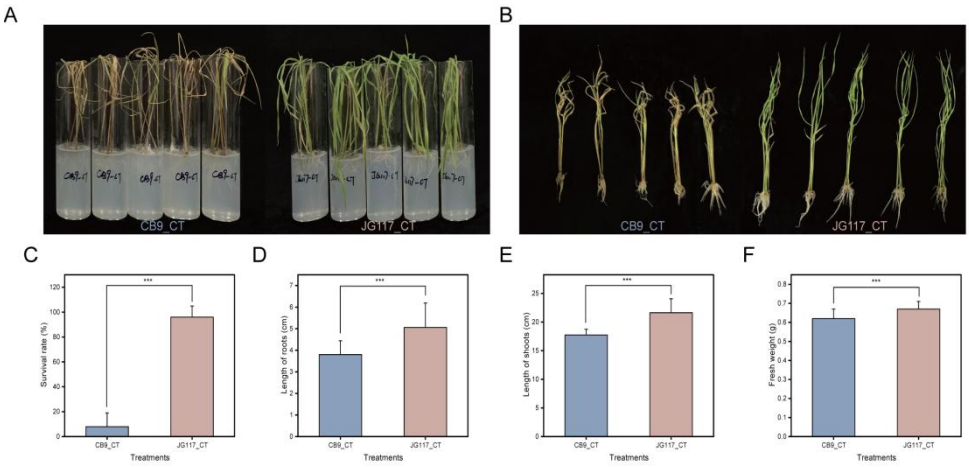

Figure 3.2 Growth performance of the rice varieties JG117 and CB9 under cold stress conditions. (A) (B) Representative images of JG117 and CB9 seedlings after 7 days of normal temperature cultivation, 7 days of cold stress, and 7 days of recovery at normal temperature. (C) Survival rates of JG117 and CB9 seedlings after cold stress treatment (CT). (D) Root length of JG117 and CB9 seedlings after cold stress treatment (CT). (E) Shoot length of JG117 and CB9 seedlings after cold stress treatment (CT). (F) Fresh weight of JG117 and CB9 seedlings after cold stress treatment (CT). Error bars represent data from five independent biological replicates. *** indicates $p < 0.05$.

These results provide further insights into the impact of cold stress on the growth

of JG117 and CB9. CB9 exhibited high sensitivity to cold stress, as evidenced by yellowing and wilting leaves and high mortality after recovery from cold stress. In contrast, JG117 demonstrated resilience to cold stress, maintaining green leaves and normal growth. Measurements of aerial and root portions confirmed that JG117 outperformed CB9 in growth parameters under cold conditions. These findings suggest that JG117 is a cold-tolerant rice variety, while CB9 is cold-sensitive.

3.3.3 Seed endophytic microbial diversity in rice varieties with different cold tolerance

To elucidate the role of endophytic microbes in rice cold tolerance, we analyzed the microbial diversity in seeds of the two rice varieties with contrasting cold tolerance: JG117 (cold-tolerant) and CB9 (cold-sensitive). Our goal was to identify key functional microbes contributing to rice cold tolerance by comparing endophytic bacterial composition, shared microbial communities, and functional microbes between the varieties.

The composition of seed-associated endophytic bacteria was analyzed by 16S rRNA V3-V4 region amplicon sequencing. Alpha diversity analysis revealed higher species richness in CB9 compared to JG117. Specifically, CB9 had an average Chao1 index of 236.29 ± 16.61, indicating higher microbial diversity than JG117, which had an average Chao1 index of 156.30 ± 30.91 (Figure 3.3A). Non-metric multidimensional scaling (NMDS) analysis further demonstrated differences in microbial community structure between JG117 and CB9 (Figure 3.3B). Heatmap analysis of the top 10 most abundant microbial taxa (at the family level) in seeds of the two varieties showed no significant differences in the abundance of Microbacteriaceae (Figure 3.3C).

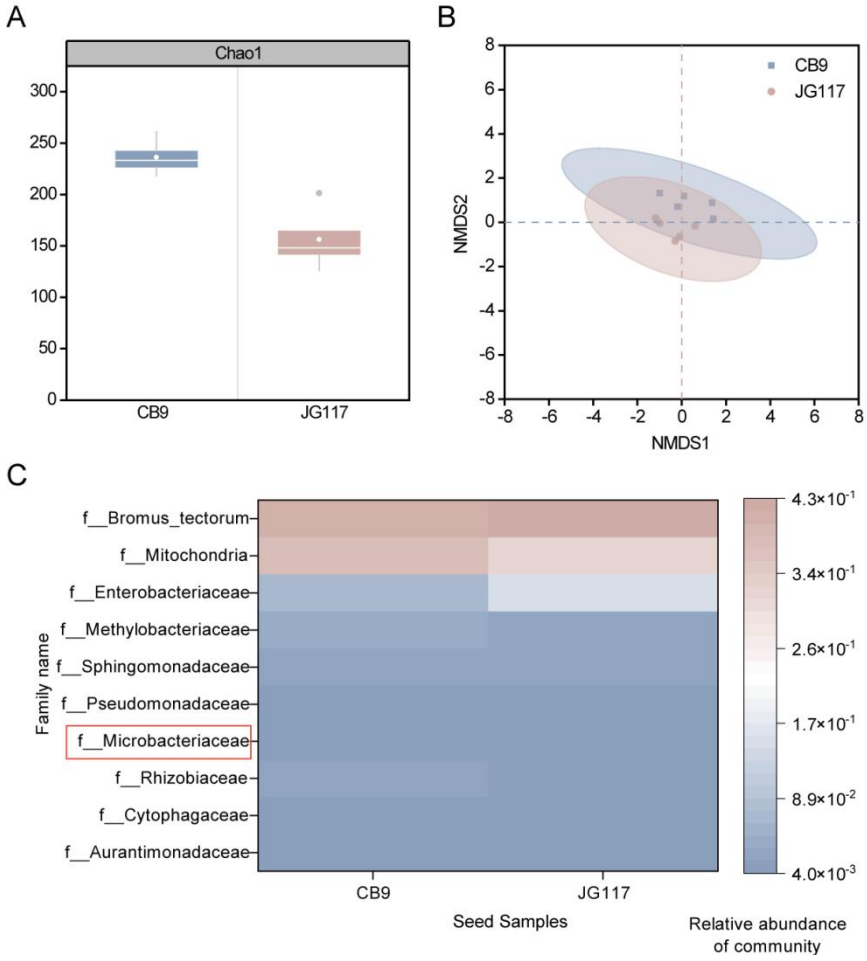

Figure 3.3 Microbial diversity analysis of seeds from rice varieties with different cold tolerances. (A) Alpha diversity index (Chao1) comparing microbial species richness in seeds of JG117 and CB9. (B) Beta diversity analysis using non-metric multidimensional scaling (NMDS) to demonstrate differences in microbial community structures between JG117 and CB9 seeds. (C) Heatmap analysis of highly abundant OTUs and their taxonomy in seeds of JG117 and CB9.

These findings demonstrate differences in microbial composition and population abundance in seeds of rice varieties with contrasting genetic backgrounds and cold tolerance. Previous studies have reported that host genotype and environmental conditions influence microbial composition. For example, host genotype and salt stress were shown to impact seed-associated microbial communities in salt-tolerant rice varieties (Walitang et al., 2018). JG117, a japonica rice variety, was bred from parental lines Changbai15 and Changbai16, while CB9 was bred from parental lines Jijing60 and Dongbei125. Differences in genetic background, cultivation history,

and environmental conditions likely contributed to the observed differences in endophytic microbial communities between the two varieties.

3.3.4 Endophytic microbial diversity in seedlings under different temperature conditions

We extended our study to the seedling stage, examining the diversity of endophytic microbes in JG117 and CB9 seedlings under different temperature conditions. Seedlings were initially grown at 26℃ for 7 days and then subjected to cold stress at 4℃ for an additional 7 days. The aim was to investigate the effects of temperature fluctuations on endophytic microbial diversity and their potential regulatory roles in rice cold tolerance.

Alpha diversity analysis (Chao1 index) of 16S rRNA sequencing data showed higher microbial diversity in JG117 seedlings under normal temperature conditions. JG117_RT (JG117 at room temperature) had a Chao1 index of 134.97 ± 67.75, higher than CB9_RT (57.14 ± 37.45) (Figure 3.4A). Under cold stress, microbial diversity increased in both varieties, with CB9_CT (CB9 under cold stress) having a slightly higher Chao1 index (660.79 ± 34.07) than JG117_CT (610.80 ± 47.91). Notably, within each variety, microbial richness increased under cold stress compared to normal conditions, potentially indicating activation of latent microbial populations under low temperatures (Castro Hector et al., 2010). These activated microbes may provide protective functions for host plants, such as secreting antifreeze substances, contributing to osmotic regulation, or activating antioxidant systems to help plants resist cold stress (Singh et al., 2020).

Beta diversity analysis via NMDS emphasized significant effects of temperature treatment (cold vs. normal) on microbial community structure, along with varietal differences (JG117 vs. CB9). The selective effects of temperature and the enrichment of specific microbial communities by rice varieties may contribute to cold tolerance. These findings highlight the importance of endophytic microbial diversity in rice adaptation to environmental changes and suggest a potential role for these microbes in rice growth and cold tolerance (Figures 3.4B and C).

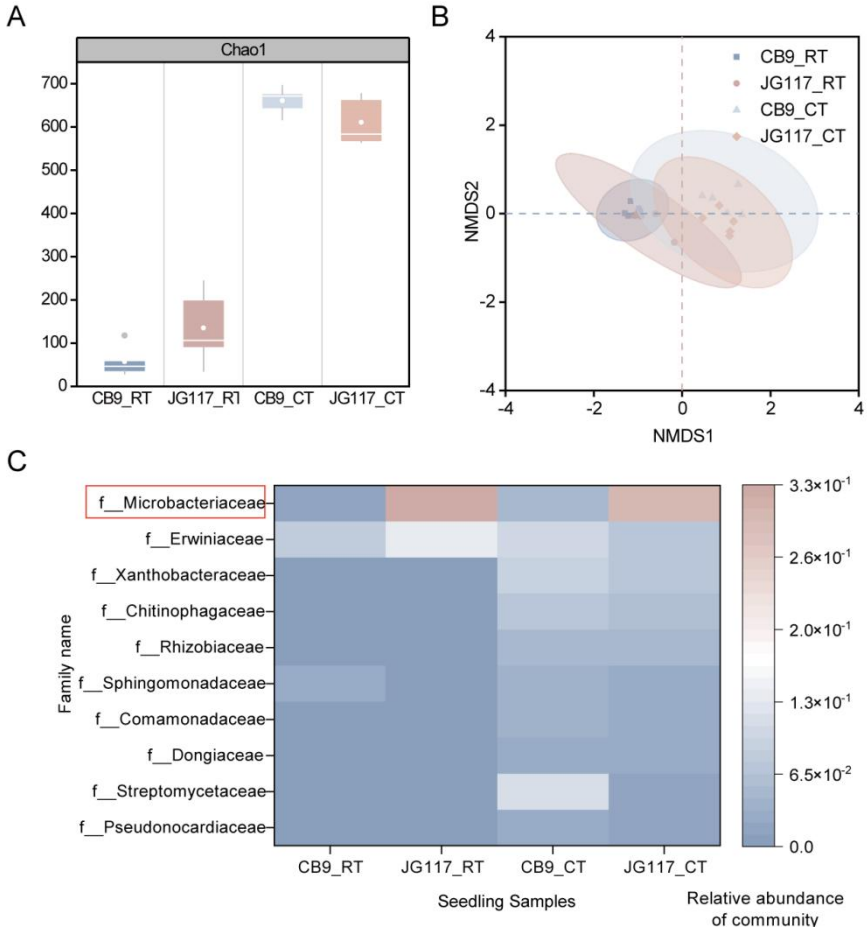

Figure 3.4 Microbial diversity analysis of seedlings from rice varieties with different cold tolerances under different temperature treatments. (A) Alpha diversity index (Chao1) of JG117 and CB9 seedlings grown under normal temperature conditions (RT) and cold stress (CT). (B) NMDS analysis of seedlings showing the effects of different temperature treatments and rice varieties on the microbial community structure of JG117 and CB9. (C) Heatmap analysis of highly abundant OTUs and their taxonomy in JG117 and CB9 seedlings under different temperature treatments.

Heatmap analysis of the most abundant OTUs and their taxonomy in seeds and seedlings revealed significant differences in Microbacteriaceae abundance in rice seedlings under different temperature treatments. Under cold stress, the abundance of Microbacteriaceae in JG117 seedlings was approximately 531.93% higher than in CB9 (0.2946 vs. 0.0466), while under normal conditions, JG117 showed a 1579.71% higher abundance than CB9 (0.3255 vs. 0.0194). This suggests that CB9 lacks the ability to effectively enrich this key microbial group, resulting in

insufficient microbial support to cope with environmental stress under cold conditions. The observed differences in microbial abundance may also reflect changes in the metabolic demands of the host plants under different environments. For example, under cold conditions, plants exhibit altered metabolic activities, such as carbohydrate accumulation and increased antioxidant enzyme activity, which may be regulated and enhanced by specific microbes. The ability of JG117 to enrich Microbacteriaceae under cold stress may contribute to its enhanced cold tolerance. Although Microbacteriaceae abundance did not differ significantly between the seeds of JG117 and CB9, its selective enrichment during seedling growth suggests a role in plant growth and cold tolerance under stress conditions.

Further analysis revealed significant changes in endophytic microbial populations within the same rice variety under different temperature treatments, with stress conditions inducing significant increases in microbial abundance. Differences in microbial population composition and abundance between rice varieties under the same temperature treatment, particularly in Microbacteriaceae abundance, may be key factors contributing to the contrasting cold tolerance observed in JG117 and CB9.

3.4 Conclusion

This chapter investigated the cold tolerance and endophytic microbial diversity of two rice varieties, JG117 (cold-tolerant) and CB9 (cold-sensitive), to elucidate the potential role of endophytic microbes in enhancing plant cold tolerance. The findings highlighted distinct growth performances and microbial community compositions between the two varieties under both normal and cold stress conditions.

Under normal temperature (26℃), JG117 and CB9 exhibited similar root growth, while JG117 showed significantly longer shoots but lower fresh weight compared to CB9. However, after exposure to cold stress (4℃) and recovery, JG117 displayed remarkable resilience, maintaining green leaves, high survival rates, and superior shoot and root growth, whereas CB9 experienced severe wilting, yellowing, and high mortality. These results confirmed JG117's superior cold tolerance compared to CB9.

Endophytic microbial diversity analysis revealed significant differences between the two varieties. CB9 exhibited higher alpha diversity in seed-associated microbial communities, but JG117 showed more distinct microbial community structures and higher enrichment of Microbacteriaceae during seedling growth, especially under cold stress. Notably, under cold conditions, Microbacteriaceae abundance in JG117 seedlings was significantly higher than in CB9, suggesting its potential role in cold stress adaptation. The ability of JG117 to selectively enrich beneficial microbial groups under cold conditions highlights the influence of host genotype and environmental conditions on endophytic microbial composition.

Overall, this chapter underscores the critical role of endophytic microbial diversity in shaping rice cold tolerance. The selective enrichment of Microbacteriaceae in

JG117 suggests that these microbes may act as key contributors to cold stress resilience. Future studies should focus on functional characterization of these microbes to uncover the underlying mechanisms by which they enhance cold tolerance, paving the way for microbe-assisted strategies to improve crop resilience in challenging climates.

Chapter 4

Isolation, identification, and functional characterization of endophytic microorganisms from seeds of cold-tolerant rice varieties

Building upon the microbial community analysis in Chapter 3, this chapter focuses on isolating and identifying endophytic microorganisms from the seeds of the cold-tolerant rice variety JG117. The chapter highlights the functional characteristics of these microorganisms, particularly their potential to promote plant growth and enhance cold tolerance. The findings from this chapter provide theoretical support for the selection of promising microbial strains for the subsequent experiments in Chapter 5, focusing on *M. testaceum* M15.

This chapter is adapted from the following published research article:

Zhao J, Liu X, Hou L, Xu G, Guan F, Zhang W, Luo H, Wu N, Yao B, Zhang C, Delaplace P, Tian J. The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa* L.). Microbiological Research **2024**;289:127908. https://doi.org/10.1016/j.micres.2024.127908

4.1 Introduction

Seeds of plants harbor a rich array of endophytic microorganisms that promote plant growth and stress tolerance through various mechanisms. Endophytic microorganisms in seeds not only have a profound effect on the host plant during the early stages of the plant's lifecycle but also influence the health and adaptability of offspring through vertical transmission. The unique microecological environment within seeds provides a stable habitat for these endophytic microorganisms (Shahzad et al., 2018). These microorganisms enter the plant either through vertical or horizontal transmission and co-evolve with the host throughout the plant's lifecycle (Wang and Zhang, 2023).

The composition and diversity of seed microbiomes are closely related to the plant's stress tolerance. Studies have shown that the diversity and community structure of endophytic bacteria in rice seeds are closely related to factors such as cultivation environment and varietal characteristics (Wang et al., 2021b). Endophytic microorganisms in seeds not only contribute to plant growth and development but also enhance the plant's tolerance to environmental stresses. During rice seed germination and early seedling stages, these endophytic microorganisms promote root growth and nutrient absorption by producing auxins (IAA), solubilizing insoluble phosphorus, and secreting siderophores, laying the foundation for healthy rice growth (Jana et al., 2022). However, current research on the functionality of endophytic microorganisms in rice seeds under cold stress is still limited, especially regarding the role of functional strains in improving rice cold tolerance.

Cold-tolerant microorganisms are those that can survive and grow under cold conditions, even below freezing. They are widely distributed in extreme environments such as polar soils, glaciers, permafrost layers, and polar lakes. In agricultural production, cold-tolerant microorganisms, as biological inoculants, show great potential in promoting crop growth and enhancing cold tolerance in cold regions (Mukhia et al., 2022; Khan et al., 2024). Some strains have been shown to improve plant growth and survival rates under low temperatures. These microorganisms maintain metabolic activity in cold environments through various mechanisms, such as adjusting the fatty acid composition of cell membranes, producing AFPs, accumulating osmolytes (such as proline and mannitol) to regulate osmotic pressure, activating antioxidant systems, and improving mineral nutrient absorption (Mishra et al., 2011a; Pathania et al., 2022). In addition, cold-tolerant microorganisms can improve plants' mineral nutrient supply and cold tolerance by secreting phosphatases, siderophores, and antifreeze proteins (Rizvi et al., 2021). However, research on cold-tolerant microorganisms in rice seeds is relatively scarce, especially in terms of screening and identifying cold-tolerant and growth-promoting functional strains from cold-tolerant rice varieties, which requires further investigation.

Based on the preliminary analysis of the diversity of endophytic microorganisms in cold-tolerant rice variety JG117 seeds, this study aims to screen and identify functional strains with cold tolerance and growth-promoting potential, with a

particular focus on the role of Microbacteriaceae members in enhancing rice cold tolerance. The specific research objectives include: isolating endophytic bacteria from the seeds of cold-tolerant rice variety JG117 and screening strains with cold-tolerant and growth-promoting characteristics. Phylogenetic classification and identification of isolated strains will be performed through 16S rRNA gene sequence comparison and analysis. Functional strains will be systematically evaluated for their IAA synthesis, phosphate solubilization ability, and siderophore production capability under cold conditions. The growth promotion and cold tolerance enhancement effects of functional strains on cold-sensitive rice variety CB9 under cold stress will also be studied.

This study is expected to reveal the potential mechanisms of cold-tolerant microorganisms, especially *Microbacterium testaceum* M15, in enhancing rice cold tolerance, providing scientific evidence for the development of environmentally friendly biological inoculants, enhancing rice cold tolerance, and achieving sustainable agricultural development. Given the important role of endophytic microorganisms in rice seedling growth and stress tolerance, in-depth research on the diversity, functional characteristics, and interaction mechanisms of these microorganisms with host plants is crucial for improving rice cold tolerance and productivity. By isolating and identifying cold-tolerant plant growth-promoting microorganisms (PGPMs) and revealing their mechanisms for promoting plant growth, this study can provide a theoretical foundation and practical guidance for developing novel biological fertilizers and bio-stimulants.

4.2 Materials and methods

4.2.1 Isolation and cultivation of endophytic microorganisms from the seeds of JG117

To investigate the microorganisms from the seeds of JG117, a cold-tolerant rice variety chosen for endophytic bacteria isolation. A precise weight of 2 g of seeds (approximately 70 grains) was measured on weighing paper. To eliminate surface-attached microorganisms, a multi-step surface sterilization protocol was applied. The seeds were soaked in 75% ethanol for 10 minutes, followed by three washes with sterile water. Subsequently, the seeds were immersed in 10% sodium hypochlorite solution for 10 minutes to eliminate residual surface microorganisms and then rinsed five times with sterile water to remove any sodium hypochlorite residue and prevent interference in subsequent endophyte isolation.

The sterilized seeds were placed in a sterile mortar and ground into a fine powder using a pestle to maximize the release of endophytic microorganisms. During grinding, 5 mL of sterile 0.9% sodium chloride (NaCl) solution solution was added to suspend the sample. After grinding, the mixture was left to stand for 15 minutes to allow the microorganisms to release and suspend fully in the liquid. The suspension was thoroughly mixed, and serial dilutions were performed using 0.9% saline solution at dilution levels of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. A 300-µL aliquot of each diluted suspension was spread onto R2A agar plates (130 cm × 130 cm).

The plates were incubated at 10℃ in a constant-temperature incubator. cold conditions were selected to simulate the cold growth environment of rice and to screen for cold-adapted endophytic bacteria. The incubation period was set at one month to allow slow-growing endophytic bacteria to fully develop. At the end of the incubation period, colonies on the plates were carefully examined, and representative colonies were selected based on morphological diversity (e.g., size, shape, color, and texture). The selected colonies were subcultured on fresh LB agar plates using streaking methods to obtain pure cultures.

All isolated endophytic bacterial strains were preserved in LB medium containing 20% glycerol and stored at -20℃ for subsequent physiological, biochemical, and molecular biological analyses. Additionally, some strains were preserved at -80℃ for long-term storage and to maintain viability.

4.2.2 Amplification and sequencing of 16S rRNA gene

For the identification of bacterial isolates, fresh cultures of the isolates were used as templates to amplify the 16S rRNA gene using primers 27F (5'-AGAGTTTGATCCTCGCT-3') and 1492R (5'-TACCTTGTTACGACTT-3'). PCR conditions included an initial denaturation at 94℃ for 5 minutes, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds, and extension at 72℃ for 1 minute, with a final extension at 72℃ for 7 minutes.

The amplified products were verified by gel electrophoresis and sent to TSINGKE Biotechnology Co., Ltd. (Beijing, China) for bidirectional sequencing. Sequencing data were analyzed and edited using SeqMan software to obtain high-quality 16S rRNA gene sequences. These sequences were compared with the NCBI GenBank database using BLAST to determine the taxonomic classification of the isolates.

4.2.3 Sequence alignment and phylogenetic analysis

For sequence alignment and phylogenetic analysis, the 16S rRNA gene sequences of the selected strains were aligned using MEGA X software with default parameter settings to ensure accurate alignment. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method. To provide a detailed depiction of the evolutionary relationships among the strains, annotations were added to the phylogenetic tree to highlight the information of different branches. The phylogenetic analysis results were visualized and optimized using Chiplt tools for clarity.

4.2.4 Analysis of cold tolerance in bacteria

To evaluate the cold tolerance of bacterial strains, the strains were cultured overnight in 3 mL LB medium at 37℃ with shaking at 200 rpm to ensure optimal growth and achieve the logarithmic growth phase. A 1% inoculum (approximately 30 μL) was transferred to fresh 50 mL LB medium and incubated at 4℃ with shaking at 200 rpm for 13 days.

During the incubation period, optical density at 600 nm (OD_{600}) was measured daily to monitor bacterial growth, and growth curves were recorded. Each strain was

tested in triplicate to ensure the reliability and reproducibility of the experimental results. OD_{600} measurements were used to evaluate the growth ability and cold tolerance characteristics of different strains under cold conditions, which are critical for screening cold-adapted endophytic bacteria.

4.2.5 Measurement of IAA production

The production of IAA by bacterial strains was evaluated as an indicator of their plant growth-promoting properties. IAA production was determined using a modified qualitative method (Gordon and Weber, 1951).

Isolated strains were cultured overnight in 3 mL LB medium to reach the logarithmic growth phase. A 1% inoculum (approximately 30 μL) was transferred to 3 mL LB medium containing 0.5 mg/mL L-tryptophan and incubated at 37℃ for 24 hours to induce IAA production. To assess IAA production under cold conditions, 30% inoculum (approximately 900 μL) was transferred to 3 mL LB medium containing 0.5 mg/mL L-tryptophan and incubated at 4℃ for 7 days to simulate cold environments.

After incubation, the cultures were centrifuged at 12,000 g for 10 minutes to collect the supernatant. A 0.5 mL aliquot of the supernatant was mixed with 0.5 mL Salkowski reagent and incubated in the dark at 25℃ for 30 minutes. Absorbance was measured at 530 nm using a SpectraMax M2 spectrophotometer (Molecular Devices). Each strain was tested in triplicate to ensure the accuracy and reliability of the results. IAA concentrations were quantified using a standard curve prepared with known concentrations of IAA (Bric John et al., 1991).

4.2.6 Measurement of phosphate solubilization ability

The phosphate solubilization ability of bacterial strains was assessed as an important trait for enhancing plant nutrient uptake, using a modified Nautiyal method (Nautiyal, 1999).

Single bacterial colonies were cultured overnight in 3 mL LB medium at 37℃ with shaking at 200 rpm. A 1% inoculum (approximately 30 μL) was transferred to 3 mL National Botanical Research Institute's Phosphate (NBRIP) medium and incubated at 37℃ with shaking at 200 rpm for 24 hours. To assess phosphate solubilization at low temperatures, a 1% inoculum was transferred to 3 mL NBRIP medium and incubated at 4℃ with shaking at 200 rpm for 7 days.

At the end of the incubation, soluble phosphate concentration in the supernatant was measured using the molybdenum blue method (Murphy and Riley, 1962). Absorbance was measured at 882 nm using a spectrophotometer, and concentrations were quantified using a standard curve prepared with known concentrations of K_2HPO_4.

4.2.7 Measurement of siderophore production

The production of siderophores by bacterial strains was assessed using a modified Schwyn and Neilands method (Schwyn and Neilands, 1987).

Single bacterial colonies were cultured overnight in 3 mL LB medium at 37℃

with shaking at 200 rpm. A 1% inoculum (approximately 30 μL) was transferred to 3 mL Chrome Azurol S (CAS) assay medium and incubated at 37℃ with shaking at 200 rpm for 24 hours. For siderophore production under cold conditions, 30% inoculum (approximately 900 μL) was transferred to 3 mL CAS assay medium and incubated at 4℃ with shaking at 200 rpm for 7 days.

After incubation, the supernatant was mixed with an equal volume of CAS reagent and incubated at room temperature for 1 hour. Absorbance was measured at 630 nm using a spectrophotometer. Siderophore production was calculated as follows: Siderophore Unit (%) = (Ar - As) / Ar × 100, where Ar is the absorbance of the reference, and As is the absorbance of the sample (Machuca and Milagres, 2003).

4.2.8 Statistical analysis

All experiments were performed with at least three biological replicates. Data were expressed as mean ± standard error (SE). Statistical analyses were conducted using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). Normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test. For multiple comparisons, one-way analysis of variance (ANOVA) was employed.

When homogeneity of variances was met, least significant difference (LSD) tests and Duncan's multiple range tests were performed for pairwise comparisons. If homogeneity of variances was not met, Tamhane's T2 test was used. Significance levels were set at $p < 0.05$. Different treatments in the results were annotated using lowercase letters (abcd) to indicate significant differences between groups. Groups with different letters were significantly different ($p < 0.05$), while those with the same letter were not significantly different.

4.3 Results and discussion

4.3.1 Isolation and identification of endophytic microorganisms from the seeds of JG117

Analysis of the cold tolerance and endophytic microbial diversity in JG117 and CB9 rice varieties suggested that JG117 seeds harbor endophytic microbial communities potentially contributing to enhanced cold tolerance. To leverage these endophytic microorganisms from cold-tolerant JG117 seeds to improve the cold tolerance of non-cold-tolerant rice varieties, we isolated and systematically identified the endophytic microorganisms from JG117 seeds.

Using cold conditions (10℃), 143 bacterial strains were isolated from JG117 seeds. Multiple sequence alignments and phylogenetic tree analysis revealed evolutionary relationships among these strains. The phylogenetic tree (Figure 4.1) demonstrates the diversity and complexity of endophytic microorganisms isolated from JG117 seeds.

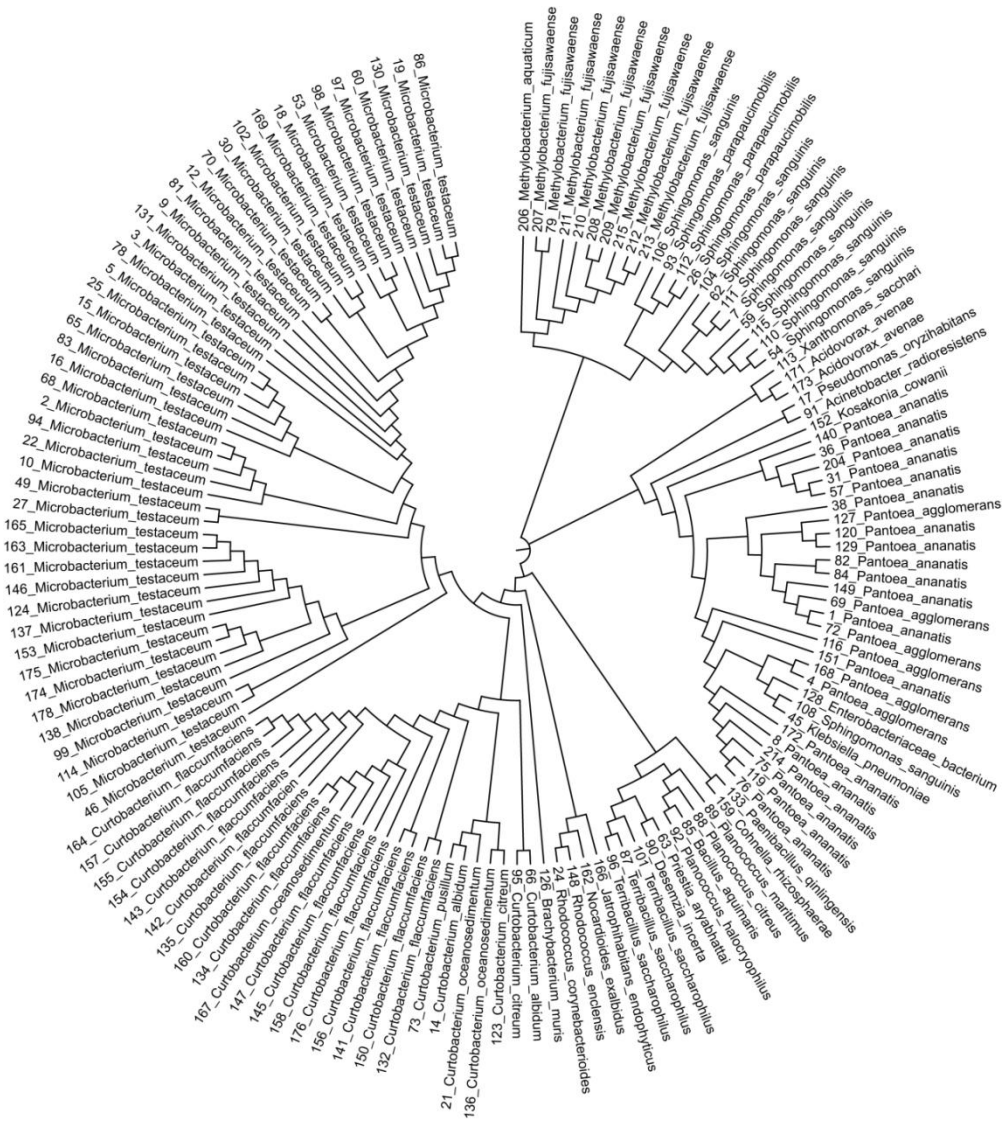

Figure 4.1 Phylogenetic tree of endophytic microorganisms isolated from JG117 seeds. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on the 16S rRNA gene sequences of 143 isolated strains, illustrating the evolutionary relationships among these isolates.

The endophytes isolated from JG117 seeds belonged to various genera, with abundant members from the Microbacteriaceae family, including *Microbacterium* and *Curtobacterium*, as well as other genera such as *Pantoea*, *Sphingomonas*, and *Methylobacterium*. Phylogenetic analysis indicated a high frequency of Microbacteriaceae members across multiple clades, highlighting their significant

role in the endophytic environment of JG117 seeds. Previous studies using high-throughput sequencing of endophytic bacterial communities in upland rice seeds revealed that genera such as *Pantoea*, *Methylobacterium*, *Sphingomonas*, and *Microbacterium* represent core microbiota commonly found in all tested upland rice seeds (Wang et al., 2021).

Within the *Microbacterium* genus, only *Microbacterium testaceum* was identified, and it was present in high abundance, indicating that it may be a dominant strain in the endophytic community of JG117 seeds. The high prevalence of *Microbacterium testaceum* suggests its potential role in promoting rice growth and stress tolerance. Additionally, several *Curtobacterium* species, including *Curtobacterium flaccumfaciens, Curtobacterium citreum*, *Curtobacterium albidum*, *Curtobacterium pusillum*, and *Curtobacterium oceanosedimentum*, were identified. However, *Curtobacterium flaccumfaciens* has been reported as a plant pathogen and was therefore excluded from further analysis in this study (Osdaghi et al., 2024).

Previous research has reported that seeds harbor various bacteria that enhance host plant growth and resilience against environmental challenges (Guha and Mandal Biswas, 2024). Endophytic bacteria in rice seeds play significant roles in plant growth, potentially allowing plants to selectively recruit beneficial microorganisms. For example, endophytic bacteria isolated from rice seeds, including *Flavobacterium* sp., *Microbacterium* sp., and *Xanthomonas* sp., have demonstrated promising plant growth-promoting activities such as hormone regulation, nitrogen fixation, siderophore production, and phosphate solubilization (Walitang et al., 2017).

These endophytic microorganisms isolated from rice seeds may have significant potential for agricultural applications as bioinoculants to improve crop cold tolerance and resilience. Our prior diversity analysis of endophytic microorganisms in seeds of different cold-tolerant rice varieties and further analysis of seedling endophytes under varying temperature treatments indicated that Microbacteriaceae members are predominant in cold-tolerant JG117, particularly under cold stress. This suggests their regulatory role in rice growth and cold tolerance. Future research will focus on the functional analysis of these Microbacteriaceae endophytes to elucidate their specific mechanisms in promoting rice cold tolerance.

4.3.2 Characterization of Microbacteriaceae microorganisms

4.3.2.1 Cold tolerance and effects on rice growth and cold tolerance at normal and low temperatures

Following the isolation, identification, and phylogenetic analysis of JG117 seed endophytes, we focused on characterizing five unique Microbacteriaceae strains: M14 (*Curtobacterium albidum*), M15 (*Microbacterium testaceum*), M21 (*Curtobacterium oceanosedimentum*), M73 (*Curtobacterium pusillum*), and M123 (*Curtobacterium citreum*). Cold tolerance assessments showed that M15 exhibited superior survival under cold conditions compared to other Microbacteriaceae strains (Figure 4.2A), indicating its strong adaptation to cold environments.

Previous research has documented the cold adaptability of Microbacteriaceae

strains isolated from extreme environments such as glacial ice, highlighting their resilience (Liu et al., 2020). For example, *Subtercola endophyticus*, isolated from surface-sterilized needles of Korean fir, thrives at temperatures between 4℃ and 25℃ (Jiang et al., 2022). Similarly, *Subtercola vilae* sp. nov., isolated from a high-altitude Chilean cold volcanic lake, grows optimally at 10-15℃ (range: 5–28℃) (Villalobos et al., 2018). However, reports on cold-adaptive *Microbacterium testaceum* are limited. Our findings expand the knowledge of cold-adapted species within Microbacteriaceae, with M15 showing robust cold tolerance.

During our investigation, we focused on the ability of M15 to enhance the cold tolerance of the cold-sensitive rice variety CB9. At the seed germination stage, rice seeds were soaked in bacterial suspension, and root irrigation was performed at the seedling stage. After cultivation under normal temperature conditions (26℃) for seven days, rice seedlings were subjected to cold stress at 4℃ for five days, followed by recovery cultivation at 26℃ for another seven days. The survival rate of M15-treated seedlings was 88.89 ± 11.11%, which was significantly higher than that of the control group (44.44 ± 11.11%), effectively doubling the survival capacity of CB9 under cold stress (Figure 4.2B). These results indicate that inoculation with M15 can improve the cold tolerance of the cold-sensitive rice variety CB9. Microbial inoculation to enhance plant cold tolerance is an effective strategy to mitigate the adverse effects of cold stress on plants. Reports on *Microbacterium testaceum* suggest its role in enhancing plant stress tolerance, particularly when isolated from various plants such as rice leaves (*M. testaceum* OsEnb-ALM-D18) (Patel et al., 2022), potato leaves(*M. testaceum* StLB037) (Morohoshi et al., 2011), and common bean leaves (*M. testaceum* BAC1065 and BAC1093) (Lopes et al., 2015). These strains have been studied as antagonistic agents to enhance plant tolerance against biotic stresses for biological control purposes. However, no reports have yet explored the role of *Microbacterium testaceum* in improving the cold tolerance of rice plants. The findings of this study reveal that *M. testaceum* mitigates the effects of cold stress in the cold-sensitive CB9 rice variety under cold conditions, providing a novel approach to improving rice cold tolerance.

In addition to evaluating the effects of bacterial inoculation on rice growth under cold conditions, we also assessed the impact under normal temperature conditions (26℃). At the seed germination stage, rice seeds were soaked in bacterial suspension, and root irrigation was performed at the seedling stage. Rice was cultivated under normal temperature conditions (26℃) for one month until reaching the three-leaf stage. The fresh weight of CB9 seedlings was then measured. Although the fresh weight of the M15-treated group (0.93 ± 0.01 g) was not the highest, it showed a significant increase compared to the control group (0.59 ± 0.08 g), highlighting its plant growth-promoting capacity (Figure 4.2C).

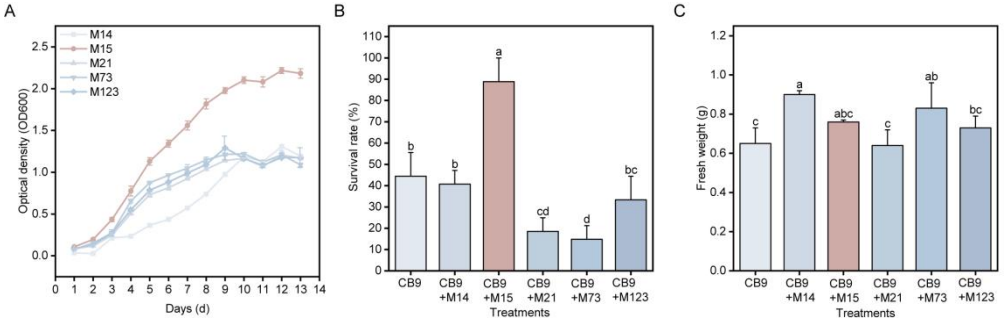

Figure 4.2 Characteristics of Microbacteriaceae strains and their effects on rice growth and cold tolerance. (A) Growth curves of Microbacteriaceae strains cultured at low temperature (4℃). (B) Survival rates of CB9 seedlings subjected to cold stress (4℃) and recovery treatment at 26℃. (C) Fresh weight of CB9 seedlings treated with different Microbacteriaceae strains under normal temperature conditions (26℃). Error bars represent data from three independent biological replicates. Means with the same letter are not significantly different at the $p < 0.05$ level. M14, *Curtobacterium albidum*; M15, *Microbacterium testaceum*; M21, *Curtobacterium oceanosedimentum*; M73, *Curtobacterium pusillum*; and M123, *Curtobacterium citreum*. CB9: CB9 rice without inoculation; CB9+M14: CB9 rice inoculated with M14; CB9+M15: CB9 rice inoculated with M15; CB9+M21: CB9 rice inoculated with M21; CB9+M73: CB9 rice inoculated with M73; CB9+M123: CB9 rice inoculated with M123.

4.3.2.2 Growth-promoting characteristics of Microbacteriaceae strains under normal temperature: analysis of IAA production, phosphate solubilization, and siderophore production

To explore the mechanisms by which the microorganisms promote rice growth, biochemical tests were conducted to evaluate the plant growth-promoting potential of M15 and other strains. The results showed that M15 exhibited significant IAA production, with a yield of 43.27±4.25 mg/mL (Figure 4.3A), which likely contributes to its plant growth-promoting properties. IAA is a naturally occurring auxin that serves as a fundamental plant hormone regulating growth and development. Studies have shown that IAA also plays a critical role in plant-microbe interactions, with beneficial bacteria utilizing IAA to promote plant growth and mitigate abiotic stresses (Etesami and Glick, 2024). For instance, *Microbacterium testaceum* Y411, isolated from *Rhynchostylis retusa* (L.) Blume, is a potent producer of IAA, which facilitates the growth and development of orchids (Yadav et al., 2022).

Additionally, M15 exhibited the highest phosphate solubilization capacity among all tested strains, with a value of 173.68±1.53 mg/L (Figure 4.3B), indicating its potential to enhance plant nutrient supply. Phosphorus is an essential macronutrient required for plant metabolism, growth, and development. PSMs facilitate the solubilization of insoluble phosphates through mechanisms such as organic acid secretion and enzyme production, making phosphates bioavailable to plants and thus

promoting growth (Rawat et al., 2021). Notably, psychrotolerant phosphate-solubilizing bacteria (PSB) have been reported as novel extremophiles for sustainable crop production in cold environments, effectively providing phosphorus to plants under such conditions (Rizvi et al., 2021).

Furthermore, all five Microbacteriaceae strains demonstrated siderophore production (Figure 4.3C), which may contribute to their plant growth-promoting ability. Iron is an indispensable micronutrient for life, and iron availability is a critical limiting factor for microbial and plant growth in agricultural environments. Certain microorganisms produce specific organic compounds, called siderophores, that chelate Fe^{3+} ions. These compounds increase and regulate iron bioavailability, thereby enhancing plant iron uptake and promoting growth (Scavino and Pedraza, 2013; Timofeeva et al., 2022). For example, *Pseudomonas* strains GRP3A and PRS9, which produce siderophores under iron-limiting conditions, have been reported to promote maize *(Zea mays* L.) growth (Sharma and Johri, 2003). In addition to promoting growth, siderophore-producing bacteria can act as biocontrol agents against plant pathogens. Siderophores derived from rhizobia have been shown to reduce oxidative damage in solanaceous plants caused by fungal pathogens (Deb and Tatung, 2024; Kumar et al., 2024).

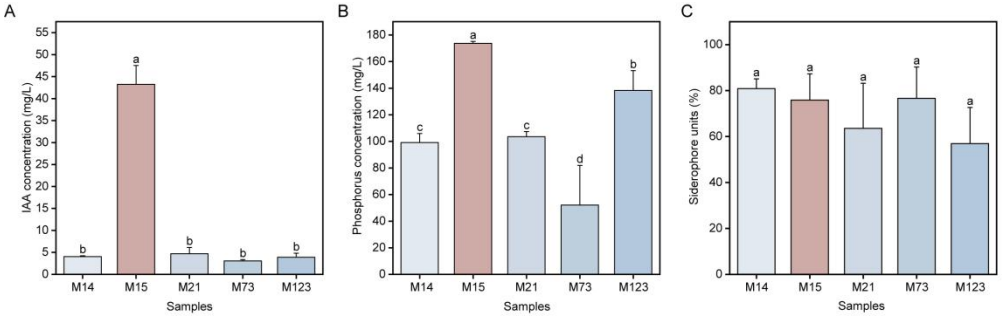

Figure 4.3 Biochemical characteristics of Microbacteriaceae strains cultured at normal temperature (37℃). (A) Indole-3-acetic acid (IAA) production of Microbacteriaceae strains cultured at 37℃. (B) Phosphate solubilization ability of Microbacteriaceae strains cultured at 37℃. (C) Siderophore production capacity of Microbacteriaceae strains cultured at 37℃. Error bars represent data from three independent biological replicates. Means with the same letter are not significantly different at the $p < 0.05$ level. M14, *Curtobacterium albidum*; M15, *Microbacterium testaceum*; M21, *Curtobacterium oceanosedimentum*; M73, *Curtobacterium pusillum*; and M123, *Curtobacterium citreum*.

4.3.2.3 Growth-promoting characteristics of Microbacteriaceae strains under cold stress: analysis of IAA production, phosphate solubilization, and siderophore production

Under cold stress conditions (4℃), M15 maintained a relatively high level of IAA production (39.94±0.74 mg/mL), which was slightly lower than its production under normal conditions (Figure 4.4A). This ability to sustain IAA production at lower temperatures likely contributes significantly to its role in promoting plant growth in

cold environments.

In terms of phosphate solubilization, M15 exhibited some reduction under cold stress, with a solubilization level of 29.18±2.16 mg/L, but it still outperformed the other tested strains (Figure 4.4B). While this represents a reduction compared to optimal temperature conditions, the sustained phosphate solubilization highlights M15's consistent ability to enhance nutrient utilization under diverse environmental conditions.

Siderophore production by M15 decreased under cold stress, consistent with trends observed for all tested strains (Figure 4.4C). Siderophores are critical for iron acquisition, especially under nutrient-limited and stress conditions. The ability of M15 to continue siderophore production, albeit at reduced levels, suggests its capability to support plant iron absorption, which is crucial for growth in cold environments.

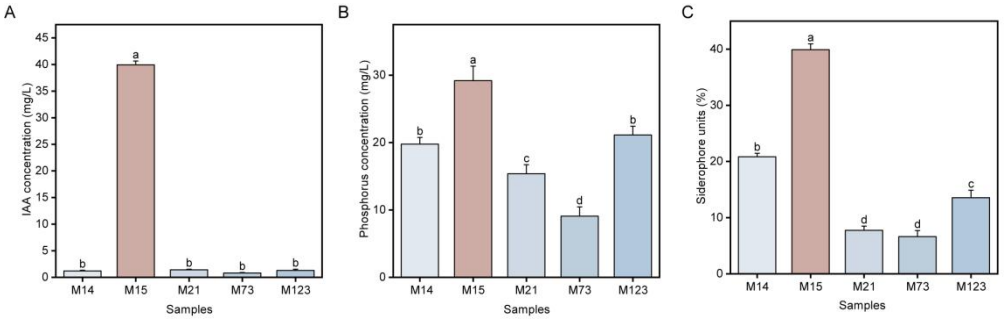

Figure 4.4 Biochemical characteristics of Microbacteriaceae strains cultured under cold-stress conditions (4℃). (A) Indole-3-acetic acid (IAA) production of Microbacteriaceae strains cultured at 4℃. (B) Phosphate solubilization ability of Microbacteriaceae strains cultured at 4℃. (C) Siderophore production capacity of Microbacteriaceae strains cultured at 4℃. Error bars represent data from three independent biological replicates. Means with the same letter are not significantly different at the $p < 0.05$ level. M14, *Curtobacterium albidum*; M15, *Microbacterium testaceum*; M21, *Curtobacterium oceanosedimentum*; M73, *Curtobacterium pusillum*; and M123, *Curtobacterium citreum*.

Overall, these results demonstrate that M15 and other Microbacteriaceae strains retain significant plant growth-promoting potential under cold stress, making them valuable candidates for enhancing plant adaptability in cold climates.

Analysis of the results indicates that *Microbacterium testaceum* M15, isolated from the cold-tolerant rice variety JG117, exhibits significant growth-promoting characteristics under both normal (37℃) and cold (4℃) conditions. These include IAA production, phosphate solubilization, and siderophore production, even though some growth-promoting factors were slightly reduced under cold stress. Despite these reductions, M15 effectively enhanced plant growth and stress tolerance, demonstrating its adaptability across varying environmental conditions. This adaptability suggests that M15 can be utilized across diverse climatic zones, making it a versatile tool for improving crop resilience in cold regions.

Given its growth-promoting ability and to cold tolerance-enhancing capacity, *Microbacterium testaceum* M15 has been selected for further investigation. Future studies will focus on understanding the mechanisms underlying its enhancement of plant cold tolerance, colonization dynamics within plant tissues, and efficacy across the entire rice growth cycle. The data presented in this study provide a solid foundation for utilizing *Microbacterium testaceum* M15 to enhance crop cold tolerance, thereby contributing to sustainable agricultural practices and productivity in challenging climatic conditions.

4.4 Conclusion

In this study, members of the Microbacteriaceae family were isolated and identified from the cold-tolerant rice variety JG117, with a focus on assessing their potential to promote plant growth and enhance cold tolerance. Five unique Microbacteriaceae strains were identified, excluding redundant isolates and known pathogens, and their cold tolerance and plant growth-promoting abilities under normal and cold conditions were evaluated. Among these strains, *Microbacterium testaceum* M15 demonstrated remarkable cold tolerance and growth-promoting properties. M15 exhibited superior cold tolerance compared to other Microbacteriaceae strains, indicating its adaptability to cold environments. Furthermore, M15 significantly enhanced the cold tolerance of the cold-sensitive rice variety CB9, doubling its survival rate under cold stress. Additionally, M15 promoted plant growth under normal temperature conditions, as evidenced by the increased biomass of rice seedlings inoculated with the strain.

Further analyses elucidated the mechanisms underlying the growth-promoting effects of the strain. In terms of plant growth-promoting traits, M15 exhibited excellent IAA production, high phosphate solubilization capacity, and significant siderophore production under normal conditions. These traits were maintained under cold stress, albeit at slightly reduced levels, indicating M15's consistent plant growth-promoting potential across different environmental conditions. These abilities are likely crucial for nutrient acquisition and plant growth promotion, contributing to enhanced cold tolerance.

In conclusion, the results of this study demonstrate that *Microbacterium testaceum* M15 possesses significant potential to promote rice growth and enhance cold tolerance. Its ability to survive and promote plant growth under cold stress makes it a valuable candidate for agricultural applications, particularly in regions vulnerable to cold climates. Future research should focus on the colonization patterns of M15 within rice plants and the detailed mechanisms of its interaction with plant hosts to further elucidate its role in enhancing crop stress tolerance.

Chapter 5

Evaluation and mechanistic analysis of *Microbacterium testaceum* M15 in promoting rice growth and enhancing cold tolerance

Based on the identification and functional evaluation of endophytic microorganisms in Chapter 4, this chapter further investigates the physiological and biochemical responses of CB9 seedlings inoculated with *M. testaceum* M15 under normal and cold conditions. The chapter analyzes the plant growth-promoting effects and cold tolerance enhancement mechanisms of *M. testaceum* M15, providing critical experimental data that inform the molecular mechanism analysis in Chapter 6.

This chapter is adapted from the following published research article:

Zhao J, Liu X, Hou L, Xu G, Guan F, Zhang W, Luo H, Wu N, Yao B, Zhang C, Delaplace P, Tian J. The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa* L.). Microbiological Research **2024**;289:127908. https://doi.org/10.1016/j.micres.2024.127908

5.1 Introduction

Microbial inoculants, as green, safe, and environmentally friendly agricultural inputs, have shown tremendous potential in improving crop stress tolerance and promoting growth in recent years (Díaz-Rodríguez et al., 2025). PGPMs have garnered widespread attention due to their potential in promoting plant growth and enhancing stress tolerance (Ansabayeva et al., 2025). PGPMs can promote plant growth and enhance tolerance to adverse environments through various mechanisms, including the synthesis of plant hormones, nitrogen fixation, phosphate solubilization, secretion of antifreeze proteins, and antioxidant enzymes.

Previous studies have shown that specific microorganisms can significantly improve plant tolerance to cold stress by enhancing nutrient absorption, regulating hormone balance, and activating defense systems. For example, *Burkholderia phytofirmans* PsJN strain has been shown to significantly promote the growth of grapevines under cold conditions (Fernandez et al., 2012). Microorganisms enhance plant tolerance to cold stress through various physiological and biochemical mechanisms, primarily involving the regulation of plant hormone balance, enhancement of antioxidant systems, secretion of antifreeze proteins, and accumulation of osmotic regulators.

Microorganisms can synthesize plant hormones such as IAA, GA, and CKs, which promote root development and plant growth, thereby enhancing the plant's ability to adapt to environmental stresses (Fernandez et al., 2012; Khedkar et al., 2024). Additionally, microorganisms can reduce ABA levels, mitigating growth inhibition under cold conditions (Singh et al., 2023; Li et al., 2025). Moreover, microorganisms can secrete ACC deaminase, which lowers ethylene accumulation in plants, alleviating the inhibitory effects of cold stress on plant growth (Koza et al., 2022). cold stress leads to the accumulation of ROS, triggering oxidative stress. Microorganisms activate the plant's antioxidant system, promoting the activity of antioxidant enzymes such as SOD, CAT, and POD, helping plants eliminate excess ROS and reducing lipid peroxidation and membrane damage (Mishra et al., 2023).

Microbacterium testaceum M15 is an endophytic bacterium isolated from rice seeds. Preliminary studies have shown that it has good growth-promoting effects under normal temperature conditions. Experimental results indicate that the M15 strain can promote root development and nutrient absorption by synthesizing plant hormones (e.g., IAA), dissolving phosphates, and producing iron carriers. Despite its significant growth-promoting effects, the potential of M15 in enhancing rice cold tolerance and its underlying mechanisms have not been thoroughly investigated.

Given the potential of the M15 strain in enhancing plant cold tolerance, this study uses the cold-sensitive rice variety CB9 as the experimental material to explore the effects of the M15 strain on rice seedling growth and cold tolerance, and to reveal its mechanisms from a physiological and biochemical perspective. First, seeds and seedlings of CB9 will be treated with M15 bacterial suspension for seed soaking and root irrigation to assess the impact of M15 on seedling growth under both normal and cold conditions. Indicators such as root length, stem length, fresh weight, seed germination rate, and seedling survival rate will be measured. Secondly, the effects

of M15 on chlorophyll content, total protein content, malondialdehyde (MDA) content, and CAT activity in rice seedlings under cold stress will be analyzed to reveal the physiological and biochemical mechanisms by which M15 enhances rice cold tolerance under stress.

This study aims to further clarify how the M15 strain enhances rice cold tolerance by regulating plant metabolism and antioxidant systems.Through this research, the ability of the M15 strain to promote rice growth and improve cold tolerance will be further evaluated, and the physiological and biochemical mechanisms by which M15 alleviates the effects of cold stress on rice will be explained. This will provide theoretical support and practical guidance for the development of novel microbial fertilizers and offer new biological strategies to enhance the cold tolerance and productivity of rice and other crops.

5.2 Materials and methods

5.2.1 Preparation of bacterial suspension

A single bacterial colony was picked from a fresh LB agar plate and inoculated into 50 mL liquid LB medium. The culture was incubated overnight at 37℃ with shaking at 200 rpm. The overnight bacterial culture was centrifuged at 5,000 × g for 10 minutes at 4℃ to minimize bacterial damage. The bacterial pellet was collected and resuspended in sterile 0.9% sodium chloride (NaCl) solution to a final concentration of 10^8 CFU/mL.

5.2.2 Treatment of rice seeds

CB9 seeds were surface-sterilized by immersion in 70% ethanol at room temperature for 3 minutes to eliminate surface microbes. The seeds were then rinsed five times with sterile distilled water to remove ethanol residues, followed by soaking in 1% sodium hypochlorite solution with shaking for 10 minutes. Afterward, the seeds were rinsed three more times with sterile distilled water to ensure thorough surface sterilization. The sterilized CB9 seeds were immersed in the prepared bacterial suspension and shaken at 120 rpm at 30℃ for 6 hours to promote bacterial adherence to the seed surface. Control group seeds were treated with sterile 0.9% sodium chloride (NaCl) solution. The treated seeds were placed on moistened sterile filter paper in Petri dishes and incubated in the dark at 30℃ for one day to promote germination.

5.2.3 Bacterial promotion of rice growth experiment

Germinated rice seeds were transplanted into pots containing sterilized vermiculite to prevent contamination by external microbes, and watered with sterile half-strength Murashige and Skoog (1/2 MS) nutrient solution to ensure adequate nutrition for the plants. The vermiculite was autoclaved at 121℃ for 20 minutes to ensure sterility. Seedlings were grown for one month under controlled conditions of 26℃ with a 14-hour light/10-hour dark photoperiod, simulating optimal growth conditions. Each seedling was irrigated with 10 mL of bacterial suspension or sterile

water (control) every three days to ensure sustained bacterial effects. Each treatment included three 9 cm × 9 cm pots, with nine seeds sown per pot. Root length, shoot length, and fresh weight were recorded to assess the growth-promoting effects of the bacterial treatment.

5.2.4 Survival rate of rice under cold stress

To evaluate the effect of M15 on the cold tolerance of rice seedlings, one-month-old seedlings cultured under normal conditions were treated with bacterial suspension or saline and then subjected to cold stress at 4℃ with a 14-hour light/10-hour dark photoperiod for five days. Following the cold stress treatment, seedlings were transferred to 26℃ for a seven-day recovery period. Each treatment included three replicates, with nine seedlings per replicate. The number of surviving seedlings was recorded after the recovery period, and the survival rate was calculated using the formula:

Survival rate (%) = (Number of surviving seedlings / Total number of seedlings) × 100%.

5.2.5 Germination rate of rice seeds under cold conditions

To assess the effect of bacterial treatment on the germination of rice seeds under cold conditions, rice seeds treated with bacterial suspension (experimental group) and sterile saline (control group) were incubated in the dark at 14℃ for seven days to simulate cold stress. Each treatment included three replicates, with 30 seeds per replicate. The number of germinated seeds was recorded to calculate the germination rate using the formula:

Germination rate (%) = (Number of germinated seeds / Total number of seeds) × 100%.

5.2.6 Chlorophyll content analysis

Chlorophyll content, an important indicator of photosynthetic capacity and plant health, was measured after cold stress treatment. Fresh rice leaves (0.1 g) were randomly collected, with three biological replicates for each treatment to ensure result reliability. Leaves were ground in an ice bath with 2 mL of extraction buffer (ethanol:acetone = 4.5:4.5:1). The mixture was centrifuged at 12,000 × g for 10 minutes at 4℃ to remove cellular debris, and the supernatant was collected. Absorbance at 663 nm and 645 nm was measured using a spectrophotometer(Arnon, 1949). Chlorophyll a, chlorophyll b, and total chlorophyll concentrations were calculated using the following formulas:

Chlorophyll a = $(12.72 \times OD_{663} - 2.59 \times OD_{645}) \times V / 1000\ W$;

Chlorophyll b = $(22.88 \times OD_{645} - 4.67 \times OD_{663}) \times V / 1000\ W$;

Total chlorophyll = $(8.05 \times OD_{663} + 20.29 \times OD_{645}) \times V / 1000\ W$,

where V is the extraction volume (mL), and W is the fresh weight of leaves (g).

5.2.7 Total protein content analysis

Total protein content, an indicator of plant growth status and stress tolerance, was measured after cold stress treatment. Fresh rice leaves (0.1 g) were collected, with three biological replicates for each treatment. Leaves were ground in an ice bath with buffer solution (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% Triton X-100, and protease inhibitors). The homogenate was centrifuged at 12,000 × g for 10 minutes at 4℃, and the supernatant was collected. The supernatant was mixed with Laemmli buffer and heated at 95℃ for 5 minutes. Protein content was determined using a BCA protein assay kit (Solarbio, China) according to Bradford's method (Bradford, 1976).

5.2.8 Malondialdehyde (MDA) content analysis

MDA, a product of lipid peroxidation, reflects oxidative stress in plant cells. Fresh rice leaves (0.5 g) were collected after cold stress treatment, with three biological replicates for each treatment. Leaves were ground in an ice bath with 5 mL of 5% trichloroacetic acid (TCA), and the homogenate was centrifuged at 12,000 × g for 10 minutes at 4℃. The supernatant (2 mL) was mixed with 2 mL of 0.5% thiobarbituric acid (TBA) and heated in a water bath at 95℃ for 30 minutes. The mixture was rapidly cooled in an ice bath, and absorbance at 532 nm, 600 nm, and 450 nm was measured. MDA content was calculated using the formula:

MDA (μmol/g FW) = $6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$ (Heath and Packer, 2022).

5.2.9 Catalase (CAT) activity analysis

CAT, a key enzyme in the antioxidant system, helps remove hydrogen peroxide and mitigate oxidative stress. Fresh rice leaves (0.1 g) were collected after cold stress treatment, with three biological replicates per treatment. Leaves were ground in an ice bath with 1 mL of phosphate buffer (50 mmol/L, pH 7.0). The homogenate was centrifuged at 12,000 × g for 10 minutes at 4℃, and the supernatant was collected. The reaction mixture contained 0.1 mL of supernatant and 0.9 mL of 0.2 mol/L H_2O_2. Absorbance changes at 405 nm were measured at 5-minute intervals using a spectrophotometer, with phosphate buffer as the control. Relative CAT activity was determined as described by Aebi (Aebi, 1984).

5.2.10 Statistical analysis

All experiments were conducted with at least three biological replicates. Data were expressed as mean ± standard error (mean ± SE). Independent sample t-tests were used to compare two groups. Statistical analyses were performed using IBM SPSS Statistics 20 software (IBM, USA). Graphs were generated using Origin 2021 Pro software (OriginLab Corporation, USA). A p-value < 0.05 was considered statistically significant.

5.3 Results and discussion

5.3.1 Effects of M15 on the growth and cold tolerance of CB9 seedlings

To investigate the impact of the M15 strain on the growth and cold tolerance of CB9 seedlings, we evaluated their growth performance under normal and cold conditions. Previous experiments demonstrated that M15 could promote the growth of rice seedlings under both conditions and enhance their cold tolerance under cold stress. To further elucidate the underlying mechanisms, a detailed analysis of growth performance and cold tolerance was conducted.

5.3.1.1 Effects of M15 on the seedling growth of CB9 under normal conditions

We evaluated the impact of M15 inoculation on the growth of rice seedlings under normal conditions. CB9 seedlings were treated with bacterial suspensions during seed germination and at the seedling stage, followed by growth under normal conditions (26℃) for one month. Measurements included root length, shoot height, and fresh weights of the aboveground and belowground parts. Results showed that M15-treated CB9 seedlings (CB9+M15) exhibited significantly better growth compared to untreated CB9 seedlings (CB9) (Figure 5.1A). Specifically, M15-treated seedlings showed increased shoot and root lengths (6.5 cm and 0.79 cm, respectively) (Figure 5.1B) and higher fresh weights (0.66 g and 0.23 g, respectively) (Figure 5.1C). These findings indicate that M15 enhances the growth of CB9 rice under normal conditions.

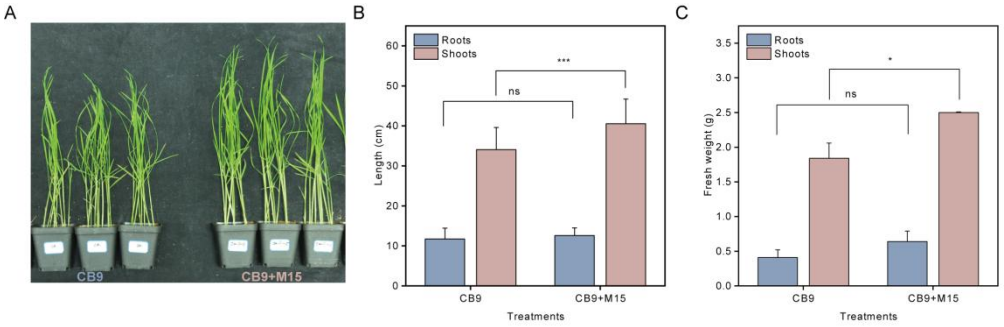

Figure 5.1 Effects of M15 treatment on the seedling growth of CB9 under normal temperature conditions. (A) Growth performance of untreated control (CB9) and M15-treated (CB9+M15) seedlings cultured at normal temperature (26℃) for one month. (B) Comparison of stem and root lengths between untreated control (CB9) and M15-treated (CB9+M15) seedlings. (C) Comparison of fresh weights of aboveground and belowground parts between untreated control (CB9) and M15-treated (CB9+M15) seedlings. Error bars represent the standard error (SE) of three independent biological replicates. ns indicates no significant difference; * indicates $p < 0.05$; *** indicates $p < 0.001$.

This result are in agreement with the previous studies demonstrating plant

growth-promoting effects of various microorganisms through modulation of plant physiological processes (Kumar et al., 2022a). For instance, *Microbacterium testosteroni* Y411, associated with orchid aerial roots, has been reported to synthesize auxins and promote plant micropropagation (Yadav et al., 2022). The growth-promoting effect of M15 is likely linked to its production of plant hormones such as IAA, phosphate-solubilizing capabilities, and siderophore production, which collectively enhance root development and overall plant growth. Thus, M15 likely stimulates endogenous growth mechanisms and improves nutrient uptake to promote rice growth.

5.3.1.2 Effects of M15 on the germination rate of CB9 seeds under cold conditions

We also assessed the impact of M15 on the germination rate of rice seeds under cold conditions (14℃). Seeds treated with bacterial suspensions (experimental group) and sterile 0.9% sodium chloride (NaCl) solution (control group) were incubated in darkness at 14℃ for seven days to simulate cold-stress germination conditions. Results revealed that M15 treatment significantly increased the germination rate of rice seeds under cold conditions, improving from 22.67% in the control group to 66.67% (Figures 5.2A and B).

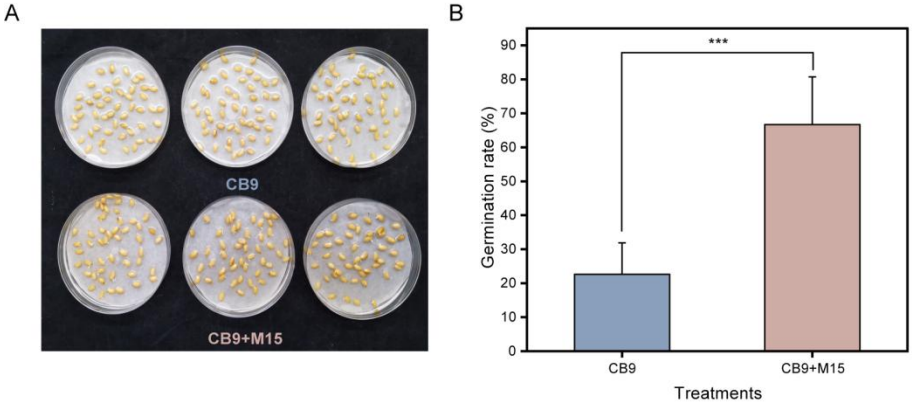

Figure 5.2 Effects of M15 treatment on the germination of CB9 seeds under cold conditions. (A) Germination performance of untreated control (CB9) and M15-treated (CB9+M15) seeds cultured at low temperature (14℃) for seven days. (B) Comparison of germination rates between untreated control (CB9) and M15-treated (CB9+M15) seeds. Error bars represent the standard error (SE) of three independent biological replicates. *** indicates $p < 0.001$.

These findings suggest that M15 alleviates the negative effects of cold stress on seed germination by enhancing seed adaptability. Similar studies have demonstrated that certain microbial strains with plant growth-promoting traits can enhance seed germination and seedling growth under stress. For example, *Microbacterium testosteroni* B2, an endophytic microorganism isolated from rice leaf tissues,

positively influences seed germination and seedling growth across different rice varieties (Patel et al., 2023). Thus, M15 is likely to enhance the physiological responses of rice seeds, thus improving seed germination under cold stress.

5.3.1.3 Effects of M15 on the survival rate of CB9 seedlings under cold stress

To evaluate the impact of M15 on the survival rate of CB9 seedlings under cold stress, seedlings initially grown at normal temperature were transferred to a 4℃ environment for five days, followed by recovery at 26℃ for seven days. Survival rates were recorded after the recovery phase. Results indicated that M15-treated seedlings exhibited significantly higher survival rates compared to the control group (Figure 5.3A). Specifically, the survival rate of M15-treated seedlings increased from 40% in the control group to 56.67% (Figure 5.3B). These findings suggest that M15 significantly enhances the cold tolerance of rice seedlings.

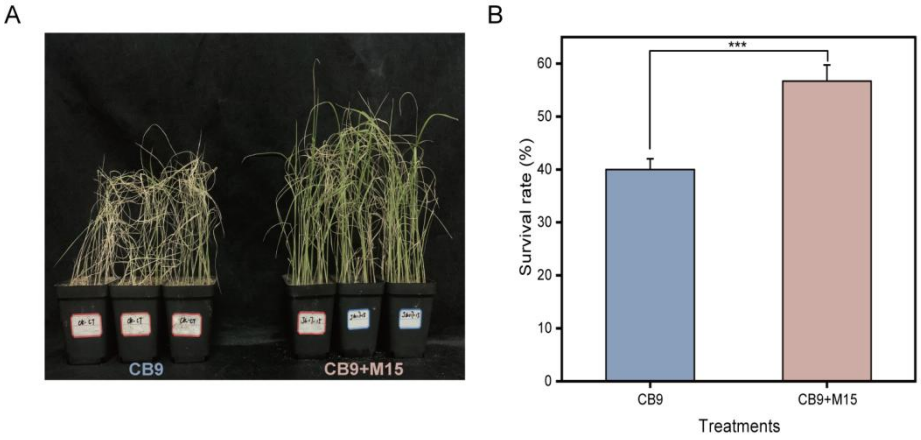

Figure 5.3 Effects of M15 treatment on the survival rate of CB9 seedlings under cold stress. (A) Growth performance of untreated control (CB9) and M15-treated (CB9+M15) seedlings after five days of cold stress (4℃) followed by seven days of recovery cultivation (26℃). (B) Comparison of survival rates between untreated control (CB9) and M15-treated (CB9+M15) seedlings. Error bars represent the standard error (SE) of three independent biological replicates. *** indicates $p < 0.001$.

This phenomenon may be attributed to the activation of cold tolerance mechanisms by M15, which helps plants mitigate the damage caused by cold stress. Previous research has shown that the rhizosphere bacterium *Burkholderia phytofirmans* PsJN can enhance cold stress tolerance in grapevines, reducing biomass loss and electrolyte leakage during chilling, while promoting recovery after stress (Barka et al., 2006). Furthermore, M15 may improve the metabolic state and antioxidant system of seedlings, mitigating cold-induced damage. For example, cold tolerance induced by *Pseudomonas* sp. strains OB155 and OS261 has been linked to enhanced germination, growth, and activation of the antioxidant defense system in

tomato plants (Subramanian et al., 2016). Similarly, M15 may enhance the cold tolerance of seedlings by stimulating the plant's antioxidant defense mechanisms, thereby improving their survival under cold stress.

5.3.2 Effects of M15 on the physiological and biochemical characteristics of CB9 seedlings under cold stress

To elucidate the mechanisms by which M15 enhances the cold tolerance of rice seedlings, the physiological and biochemical characteristics of CB9 seedlings under cold stress were comprehensively analyzed. Previous experiments demonstrated that M15 inoculation significantly improved the growth and survival rates of rice seedlings under low temperatures, particularly during the recovery phase following cold stress. To further explore the physiological basis for the enhanced cold tolerance, we examined the effects of M15 on chlorophyll content, total protein content, MDA content, and CAT activity in rice seedlings under cold stress.

5.3.2.1 Effects of M15 on chlorophyll content under cold stress

Chlorophyll is a critical component of photosynthesis, and its content reflects the photosynthetic capacity of plants under stress conditions. Following cold stress treatment, the chlorophyll a, chlorophyll b, and total chlorophyll content of CB9 seedlings treated with M15 were measured and compared to untreated controls. Results showed that M15 significantly increased chlorophyll a by 30.45%, chlorophyll b by 35.78%, and total chlorophyll content by 32.45% compared to the control group (Figure 5.4A). These findings suggest that M15 effectively maintains chlorophyll levels under cold conditions, likely by protecting photosynthetic machinery to enhance cold tolerance. Studies have reported that cold stress significantly reduces chlorophyll content, impairing photosynthesis (Barka et al., 2006). However, microbial inoculation can mitigate these adverse effects, partly by increasing photosynthetic pigment levels and regulating chlorophyll biosynthesis pathways, thus enhancing photosynthesis. For example, *Burkholderia* strain PsJN increased photosynthetic pigment levels and alleviated cold damage by improving photosynthesis and cellular morphology after nighttime stress (Su et al., 2015). Therefore, M15 likely promotes photosynthesis under cold conditions, thereby improving the growth and survival rates of rice seedlings.

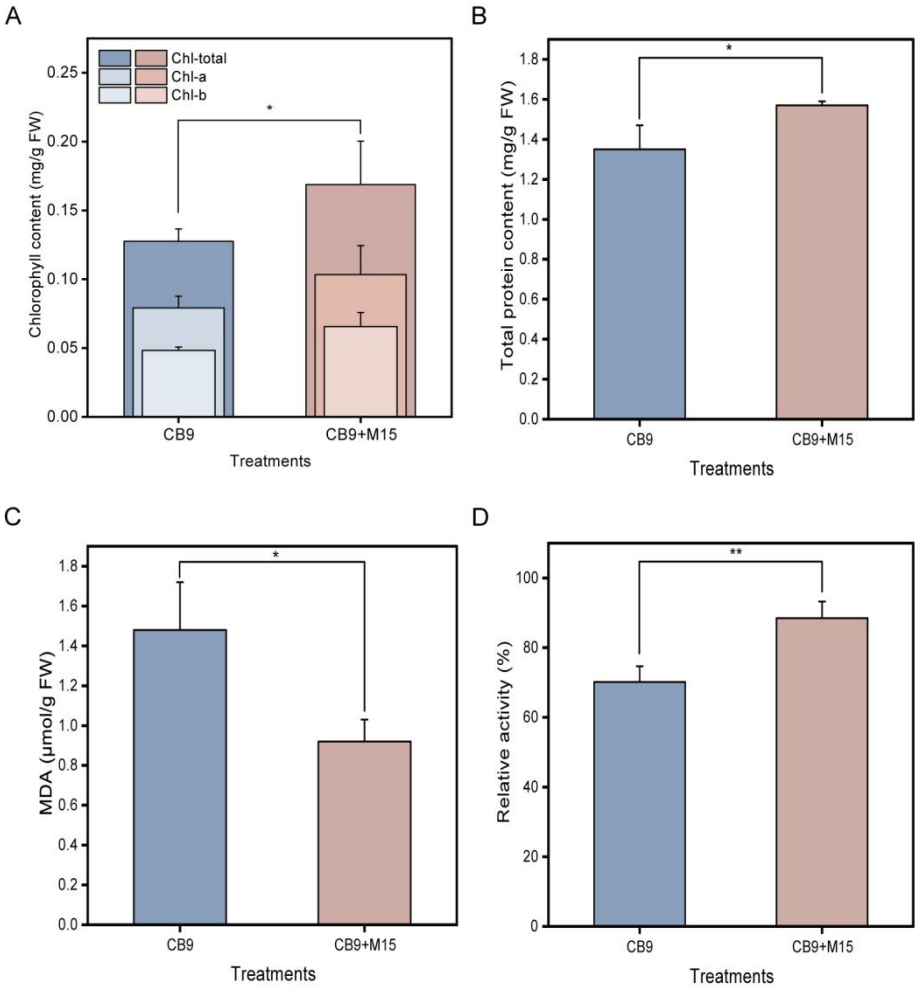

Figure 5.4 Effects of M15 treatment on the physiological and biochemical characteristics of CB9 seedlings under cold stress. (A) Comparison of chlorophyll a, chlorophyll b, and total chlorophyll content between untreated control (CB9) and M15-treated (CB9+M15) seedlings. (B) Comparison of total protein content between untreated control (CB9) and M15-treated (CB9+M15) seedlings. (C) Comparison of malondialdehyde (MDA) content between untreated control (CB9) and M15-treated (CB9+M15) seedlings. (D) Comparison of catalase (CAT) activity between untreated control (CB9) and M15-treated (CB9+M15) seedlings. Error bars represent the standard error (SE) of three independent biological replicates. * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.2.2 Effects of M15 on total protein content under cold stress

Changes in total protein content reflect the synthesis and accumulation of proteins

under stress conditions. Under cold stress, total protein content in M15-treated rice seedlings was significantly higher than in the control group, with a 16.31% increase (Figure 5.4B). This indicates that M15 promotes protein synthesis and accumulation, enhancing the metabolic functions and survival of rice seedlings under cold stress. Previous studies have shown that plants adapted to cold conditions exhibit higher soluble protein content and greater cold tolerance (Atici et al., 2003). For instance, cold stress alleviation in wheat seedlings inoculated with cold-tolerant *Pseudomonas* strains from Ladakh resulted in significantly increased soluble protein and free amino acid content, potentially reducing the plants' sensitivity to cold (Mishra et al., 2011).

5.3.2.3 Effects of M15 on Malondialdehyde (MDA) content under cold stress

MDA is a product of lipid peroxidation and reflects the degree of oxidative damage to cellular membranes. Under cold stress, the MDA content in M15-treated CB9 seedlings was significantly lower than that in the control group (Figure 5.4C). Specifically, M15 treatment reduced MDA levels by 37.76%, indicating that M15 minimizes oxidative damage by reducing lipid peroxidation. This protection helps maintain cell membrane integrity, alleviating cold-induced damage to cell membranes and enhancing cold tolerance. These findings align with prior studies showing that microorganisms can mitigate oxidative damage by modulating plants' antioxidant systems and protecting cell membrane integrity (Kakar et al., 2016; Tiryaki et al., 2019; Shi et al., 2024). M15 likely enhances antioxidant enzyme activity, helping rice seedlings eliminate excess reactive oxygen species (ROS), reduce oxidative damage, and improve cold tolerance.

5.3.2.4 Effects of M15 on Catalase (CAT) activity under cold stress

Catalase (CAT) is a key antioxidant enzyme that decomposes hydrogen peroxide, mitigating oxidative stress. Under cold stress, CAT activity in M15-treated CB9 seedlings was significantly higher than in the control group, with a 26.15% increase (Figure 5.4D). This suggests that M15 activates plant antioxidant mechanisms by enhancing CAT activity, helping plants eliminate hydrogen peroxide generated under cold stress and reducing oxidative damage. Increased CAT activity helps maintain ROS homeostasis in cells, thereby improving the stress tolerance of rice seedlings under low temperatures. Previous studies have demonstrated that microorganisms enhance plant antioxidant capacity under cold stress by promoting the activity of endogenous antioxidant enzymes (Tiryaki et al., 2019). For example, three plant growth-promoting rhizobacteria (PGPR) strains (*Bacillus subtilis* AR156, *Bacillus amyloliquefaciens* SM21, and *Serratia* sp. XY21) improved cold tolerance in tomato seedlings by enhancing antioxidant defense systems (Wang et al., 2016). Similarly, cold-tolerant endophytic *Pseudomonas* strains OB155 and OS261 induced cold tolerance in tomato (*Solanum lycopersicum* Mill.) by activating antioxidant capacity (Subramanian et al., 2015). Therefore, M15 likely enhances the antioxidant system of rice seedlings, helping them adapt to cold stress and improving their survival.

5.4 Conclusion

This study comprehensively explored the role of *M. testaceum* M15 in promoting rice seedling growth and enhancing cold tolerance, along with its underlying mechanisms. The findings demonstrate that M15 significantly promotes rice seedling growth and improves its adaptability to cold stress through multiple pathways.

Firstly, M15 treatment markedly increased the root length, shoot height, and fresh weight of CB9 seedlings under normal temperature conditions, highlighting the strain's notable plant growth-promoting effects. These effects may be attributed to the production of plant growth hormones (e.g., IAA), phosphate solubilization, and siderophore production by M15, which enhance rice root development and nutrient absorption, thereby supporting seedling health and growth. Additionally, M15 significantly improved seed germination rates and seedling survival under cold stress, indicating its crucial role in mitigating the adverse effects of cold conditions on rice seeds and seedlings.

Physiological and biochemical analyses further elucidated the mechanisms by which M15 improves rice cold tolerance. M15 treatment significantly increased chlorophyll content, total protein content, and CAT activity in seedlings, while significantly reducing the accumulation of MDA. These results indicate that M15 enhances the photosynthetic capacity and antioxidant system of rice, thereby reducing the damage caused by cold stress to cell membranes and metabolic activities. Moreover, the increased CAT activity facilitates the removal of ROS induced by cold stress, protecting rice cells from oxidative damage. These mechanisms collectively promote the growth and survival of rice seedlings in cold environments.

In conclusion, *M. testaceum* M15 regulates plant physiological and biochemical processes to not only enhance rice seedling growth under normal conditions but also effectively mitigate the adverse effects of cold stress by boosting antioxidant system activity and reducing oxidative damage. These findings provide a scientific foundation for the use of M15 as a potential plant-growth-promoting microorganism with agricultural applications and further support its practical potential in improving crop cold tolerance and productivity.

Chapter 6

Genomic and transcriptomic insights into the molecular mechanisms underlying the role of *Microbacterium testaceum* M15 in enhancing rice cold tolerance

Building on the physiological and biochemical data from Chapter 5, this chapter explores the genomic and transcriptomic characteristics of *M. testaceum* M15. It examines key genes related to cold adaptation and plant growth promotion, integrating genomic and transcriptomic analysis to uncover the mechanisms by which *M. testaceum* M15 influences rice under different temperature conditions. This chapter complements the experimental data from Chapter 5 and provides deeper insights into the molecular mechanisms at play.

This chapter is adapted from the following published research article:

Zhao J, Liu X, Hou L, Xu G, Guan F, Zhang W, Luo H, Wu N, Yao B, Zhang C, Delaplace P, Tian J. The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa* L.). Microbiological Research **2024**;289:127908. https://doi.org/10.1016/j.micres.2024.127908

6.1 Introduction

With the advancement of high-throughput sequencing technology, genomics and transcriptomics have become important tools for unveiling the mechanisms of microbial-plant interactions. Whole genome sequencing allows for a comprehensive understanding of the functional gene composition of microorganisms, providing insight into their genetic background and potential growth-promoting and stress-resistant functional genes. Transcriptomic analysis, on the other hand, reveals the gene expression changes of microorganisms under specific conditions and their regulatory mechanisms on plant growth and stress tolerance.

Cold-tolerant PGPB typically have complex and unique genome structures, which provide a genetic foundation for their survival in extreme environments and their growth-promoting effects on plants. Genomic studies can reveal how these microorganisms enhance plant cold tolerance through functional genes and metabolic pathways. Previous studies have shown that cold-tolerant PGPB carry key genes that help them survive cold stress. Cold shock proteins (CSPs) and heat shock proteins (HSPs) help maintain cellular structure and function under temperature fluctuations, protecting plants from cold stress (Dasila et al., 2022; Goyal et al., 2022). Antioxidant-related genes (such as SOD, CAT) increase antioxidant enzyme activity in plants, scavenging ROS produced under cold stress and reducing oxidative damage (Shen et al., 2021; Hualpa-Cutipa et al., 2022). EPS synthesis genes form biofilms, improving the rhizosphere microenvironment and enhancing plants' ability to adapt to cold, drought, and other stressors (Bhagat et al., 2021). Synthesis genes for osmoprotectants, such as betaine, proline, and trehalose, help maintain cell osmotic pressure and stability (Singh et al., 2022a; Singh, 2022). Plant hormone synthesis genes, including those for IAA, GA, and CKs, promote plant growth and root development (Timofeeva et al., 2024). The presence of these functional genes allows cold-tolerant PGPB to possess the potential to regulate plant stress tolerance under harsh environmental conditions.

Phosphorus is an essential nutrient for plant growth and metabolism, but most phosphorus in soils exists in insoluble inorganic or organic forms, making it difficult for plants to absorb directly (Khan et al., 2023c). PSMs convert insoluble phosphorus in the soil into plant-available forms, significantly improving plant nutrient absorption and growth. Phosphate solubilization mechanisms mainly include organic acid secretion, phosphatase and phospholipase secretion, and phosphate transport system modulation (Pho and Pst systems). Many PSMs secrete organic acids (such as gluconic acid, citric acid) to lower soil pH and dissolve phosphate minerals (Pan and Cai, 2023). Additionally, microorganisms can secrete acidic phosphatases, alkaline phosphatases, and phospholipases to decompose organic phosphorus compounds and release absorbable inorganic phosphorus (Iftikhar et al., 2024). Microorganisms can also regulate phosphorus absorption and metabolism through the Pho regulon and Pst system to adapt to low-phosphorus environments (Zhao et al., 2022).

In previous studies, we found that the *Microbacterium testaceum* M15 strain not only significantly promotes rice seedling growth but also effectively enhances rice

cold tolerance under cold stress. However, the molecular mechanisms through which the M15 strain enhances rice cold tolerance and growth promotion, especially at the genomic and transcriptomic levels, as well as its functional genes and metabolic pathways, have yet to be fully explored. Therefore, in-depth research into the genomic characteristics and transcriptional regulatory mechanisms of the M15 strain, revealing its functional genes and metabolic pathways in promoting rice growth and enhancing cold tolerance, is of great significance for a comprehensive understanding of its mechanisms and the development of new microbial fertilizers.

This study aims to use genomic sequencing and transcriptomic analysis to investigate the gene expression characteristics of the M15 strain under both normal and cold stress conditions, identify cold tolerance-related functional genes, and analyze their mechanisms of action on rice growth and cold tolerance. Through whole-genome sequencing and annotation, the genomic structure of the M15 strain will be analyzed, and functional genes related to cold adaptation, plant growth promotion, and stress tolerance will be identified. The gene expression differences of M15 under normal temperature (37℃) and cold (4℃) conditions will be compared, and key genes induced by low temperatures will be selected to reveal their potential mechanisms in enhancing rice cold tolerance. Combined with NBRIP medium experiments and gluconic acid content measurement, the ability of M15 to enhance phosphorus utilization through organic acid secretion will be verified. Further, exogenous phosphorus supplementation experiments will be conducted to verify the role of phosphorus metabolism in the ability of M15 to enhance rice cold tolerance. Ultimately, the molecular mechanisms through which M15 promotes rice cold tolerance will be revealed at the genomic and transcriptomic levels, and the strain's phosphate-solubilizing ability and its role in alleviating rice cold stress will be clarified..

6.2 Materials and methods

6.2.1 Bacterial genome sequencing

A single colony of *M. testaceum* M15 was inoculated into 50 mL of LB medium and cultured overnight at 37℃ with shaking at 200 rpm. After centrifugation at 5,000 g for 10 minutes at 4℃, the bacterial pellet was collected for genomic DNA extraction. The construction of the genomic library and sequencing were performed by Allwegene Technology Co., Ltd. (Beijing, China), using both third-generation Nanopore sequencing and second-generation Illumina sequencing platforms, with a sequencing depth exceeding 100×. Genome functional annotation included Gene Ontology (GO) analysis via Blast2GO, COG analysis using EggNOG-mapper, and KEGG pathway analysis via the KEGG Automatic Annotation Server. The complete genome sequence of *M. testaceum* M15 has been deposited in the NCBI GenBank database (accession number CP143563, BioProject number PRJNA1069338, BioSample number SAMN39615697).

6.2.2 Bacterial transcriptome sequencing

To investigate the gene expression profile of M15 under different temperature conditions, a single colony of M15 was inoculated into 3 mL of LB medium and cultured overnight at 37℃ with shaking at 200 rpm. A 1% inoculum was then transferred to 50 mL of LB medium and cultured until the OD_{600} reached approximately 1.0 at either 37℃ or 4℃. Three biological replicates were performed for each condition to ensure data reproducibility and reliability. Transcriptome sequencing was conducted using the Illumina HiSeq 4000 platform with paired-end (PE) 150 sequencing by Allwegene Technology Co., Ltd. (Beijing, China). Sequencing data are available in the NCBI Sequence Read Archive (accession number PRJNA1069338).

6.2.3 Measurement of pH changes in NBRIP medium

A single colony of M15 was inoculated into 3 mL of LB medium and cultured overnight at 37℃ with shaking at 200 rpm. A 1% inoculum was then transferred to 50 mL of NBRIP medium, with un-inoculated NBRIP medium serving as the control. Each treatment was conducted in triplicate. After 24 hours of shaking culture at 37℃ and 200 rpm, the cultures were centrifuged at 12,000 g for 10 minutes, and the pH of the supernatant was measured using a digital pH meter.

6.2.4 Detection of gluconic acid production

A single colony of M15 was inoculated into 3 mL of LB medium and cultured overnight at 37℃ with shaking at 200 rpm. A 1% inoculum was then transferred to 200 mL of NBRIP medium, with un-inoculated medium serving as the control. The cultures were incubated at 37℃ and 200 rpm for 24 hours, followed by centrifugation at 12,000 g for 10 minutes. A 500 μL aliquot of the supernatant was mixed with 500 μL of methanol and filtered through a 0.22 μm membrane. Gluconic acid was analyzed using liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ). Standard gluconic acid solutions were prepared by dissolving 100 mg of gluconic acid in 1 mL of ultrapure water and serially diluting to a final concentration of 1 mg/L. The LC-QQQ analysis was performed using an Agilent 1290 Infinity II LC system coupled with an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies). Chromatographic separation was achieved using a Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm, Thermo Fisher Scientific). The mobile phase consisted of water with 0.1% formic acid and acetonitrile with 0.1% formic acid under gradient elution at a flow rate of 0.2 mL/min. The injection volume was 10 μL. Mass spectrometry conditions included negative ion electrospray ionization (ESI) at a voltage of 3500 V, a drying gas flow rate of 10 L/min, a drying gas temperature of 250℃, and a nebulizer pressure of 35 psi. Detection was performed in multiple reaction monitoring (MRM) mode for the transition m/z 194.9 → 75.0, with precursor ion (Da) 194.9, product ion (Da) 75.0, dwell time 12 ms, fragmentor voltage 80 V, and collision energy 7 V (Wan et al., 2019; Zhang et al., 2024).

6.2.5 Phosphorus supplementation experiment in rice

Surface-sterilized CB9 seeds were sown in sterile vermiculite pots and grown in a controlled growth chamber at 26℃ with a 14-hour light/10-hour dark photoperiod. Phosphate solutions were prepared by dissolving P_2O_5 in deionized water at concentrations of 0, 0.16, 0.32, 0.48, and 0.64 g/L. Seedlings were irrigated with these solutions and subjected to cold stress at 4℃ for 5 days, followed by recovery at 26℃ for 7 days. Survival rates were recorded and calculated as follows: Survival rate (%) = (number of surviving seedlings / total number of seedlings) × 100%. Each treatment included three replicates, with each replicate consisting of individual seedlings.

6.2.6 Analysis of soluble phosphorus content in leaves

Surface-sterilized CB9 seeds were treated with bacterial suspensions (via seed soaking and root irrigation) as previously described. Seedlings were grown under controlled conditions at 26℃ with a 14-hour light/10-hour dark photoperiod until the three-leaf stage. Each treatment included three replicates. Approximately 0.5 g of rice seedling leaves was ground into a fine powder using liquid nitrogen, homogenized with 10 mL of sterile deionized water, and centrifuged at 12,000 g for 10 minutes at 4℃. Soluble phosphorus content in the supernatant was determined using the molybdenum blue method, with quantification based on a standard curve generated from known concentrations of K_2HPO_4 (Murphy and Riley, 1962).

6.2.7 Statistical analysis

All experiments were conducted with at least three biological replicates, and data were expressed as mean ± standard error (mean ± SE). Independent sample t-tests were used for comparisons between two groups. Statistical analyses were performed using IBM SPSS Statistics 20 software (IBM, USA). Graphs were generated using Origin 2021 Pro software (OriginLab Corporation, USA). A p-value of less than 0.05 was considered statistically significant.

6.3 Results and discussion

6.3.1 Genomic and transcriptomic insights into the mechanisms of M15 in enhancing rice cold tolerance and growth

To investigate the molecular mechanisms by which *M. testaceum* M15 enhances rice cold tolerance and promotes growth, comprehensive genomic sequencing and transcriptomic analyses were performed under normal and cold conditions. These analyses revealed functional genes and metabolic pathways related to cold adaptation, plant growth promotion, and stress tolerance. The results demonstrate that M15 has significant potential to mitigate cold stress and improve rice growth and resilience through multiple mechanisms. These findings provide important clues for further exploration of M15's potential to enhance plant growth and cold tolerance.

6.3.1.1 Genomic analysis of M15

Whole-genome sequencing of *M. testaceum* M15 yielded a complete genome sequence (NCBI GenBank accession number: CP143563; BioProject: PRJNA1069338; BioSample: SAMN39615697). The genome consists of a single circular chromosome with a total length of 3,592,971 bp and an average G+C content of 69.95% (Figure 6.1). It includes 3414 predicted genes, comprising 3344 protein-coding genes, 49 tRNA genes, 3 each of 23S rRNA, 16S rRNA, and 5S rRNA genes, 1 tmRNA gene, and 1 other RNA gene. The high G+C content suggests that M15 may have a strong tolerance to environmental stress, as genomes with higher G+C content tend to be more stable and better equipped to withstand environmental changes.

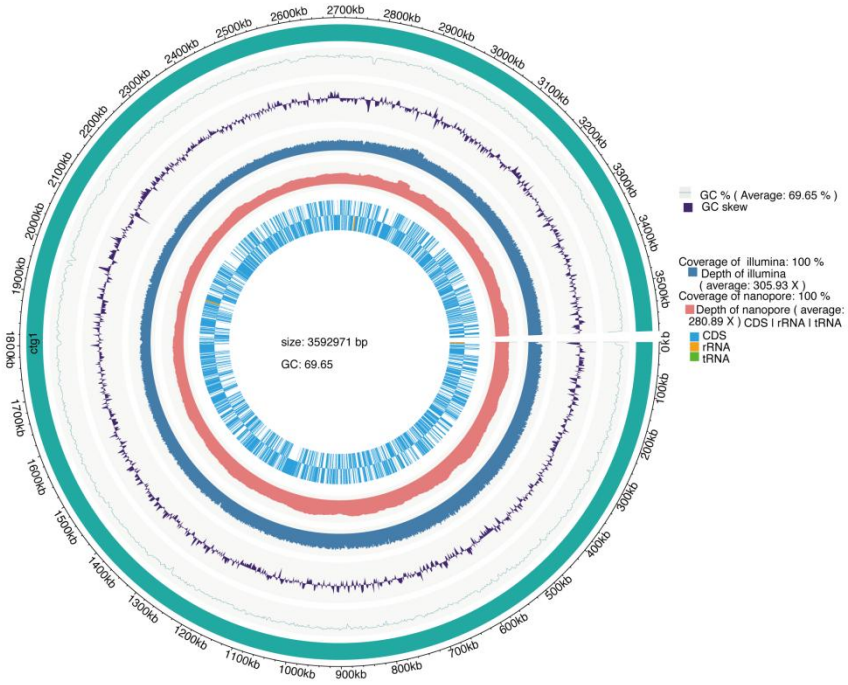

Figure 6.1 Circular genome map of *M. testaceum* M15. This figure displays the complete circular genome structure of *M. testaceum* M15, providing a comprehensive view of its general genomic features, including structural organization, GC content variation, sequencing coverage, and the distribution of coding and non-coding RNA regions.

Functional annotation and further analysis revealed multiple genes in the M15 genome associated with environmental adaptation, plant growth promotion, and enhanced cold tolerance, aiding the bacterium in mitigating cold stress and

supporting plant growth (Table 6.1). For example, genes encoding cold shock proteins (CSPs), heat shock proteins (HSPs), and molecular chaperones were identified. These genes help maintain cellular structural integrity and metabolic activity during drastic temperature changes, reducing cellular damage caused by cold stress and enhancing bacterial survival (Shen et al., 2021; Dasila et al., 2022; Singh et al., 2022). M15 synthesizes cold shock proteins (CSPs) to withstand low-temperature stress and maintain stable colonization within the host. As a result of this persistent colonization, the strain can continuously secrete plant growth-promoting compounds such as IAA and siderophores, thereby indirectly enhancing the host's cold tolerance and growth performance.

Table 6.1 Functional gene classification and annotation of genes related to cold adaptation and tolerance in the genome of *M. testaceum* M15.

Category	gene_idGene ID	Gene name	Protein coded by the gene
Cold Shock Proteins, Heat Shock Proteins, Molecular Chaperones	assembly_00566	*CspA*	cold-shock protein
	assembly_01788	*CspA*	cold-shock protein
	assembly_02875	*CspD*	cold shock domain-containing protein
	assembly_02882	*CspA*	cold-shock protein
	assembly_01545	*RbfA*	Ribosome-binding factor A
	assembly_01544	*IF2*	Translation initiation factor IF-2
	assembly_01543	*NusA*	Transcription termination/antitermination protein *NusA*
	assembly_01583	*PNPase*	Polyribonucleotide nucleotidyltransferase
	assembly_01030	*IF1*	Translation initiation factor IF-1
	assembly_01544	*IF2*	Translation initiation factor IF-2
	assembly_01621	*IF3*	Translation initiation factor IF-3
	assembly_02256	*tig*	Trigger factor
	assembly_03016	*RecA*	Recombinase *RecA*
	assembly_00538	*gyraA*	DNA gyrase subunit A
	assembly_02097	*gyraA*	DNA gyrase subunit A
	assembly_	*aceE*	Pyruvate dehydrogenase E1

	02127		component
	assembly_01956	*RbpA*	RNA polymerase-binding protein *RbpA*
	assembly_00546	*PPIase*	peptidylprolyl isomerase
	assembly_01370	*yfiA*	Ribosome hibernation promotion factor
	assembly_00313	*DeaD*	ATP-dependent RNA helicase *DeaD*
	assembly_02497	*CshA*	DEAD-box ATP-dependent RNA helicase *CshA*
	assembly_02881	*GroEL*	60 kDa chaperonin, chaperonin *GroEL*
	assembly_01104	*GroES*	10 kDa chaperonin, co-chaperone *GroES*
	assembly_03167	*HtpG*	Chaperone protein *HtpG*
	assembly_00894	*FtsH*	ATP-dependent zinc metalloprotease *FtsH*
	assembly_00309	*Putative heat shock protein HspR`*	Putative heat shock protein *HspR*
	assembly_00310	*DnaJ 1*	Chaperone protein *DnaJ 1*
	assembly_00311	*GrpE*	nucleotide exchange factor protein *GrpE*
	assembly_00312	*DnaK*	Chaperone protein *DnaK*
	assembly_02195	*DnaJ*	Chaperone protein *DnaJ*
	assembly_02196	*HrcA*	Heat-inducible transcription repressor *HrcA*
	assembly_02706	*DnaK*	Chaperone protein *DnaK*
	assembly_00284	*ClpB*	Chaperone protein *ClpB*
	assembly_01853	*Sod2*	Superoxide dismutase [Mn]
	assembly_01357	*HPI*	catalase/peroxidase *HPI*
	assembly_01795	*katG*	Catalase
Antioxidant Genes	assembly_01081	*Ohr*	Organic hydroperoxide resistance protein
	assembly_01517	*osmC*	OsmC family peroxiredoxin
	assembly_01589	*osmC*	OsmC family peroxiredoxin

	assembly_00523	*Txn*	Thioredoxin
	assembly_01378	*tpx*	thioredoxin-dependent thiol peroxidase
	assembly_00522	*TRXR*	Thioredoxin reductase
	assembly_01177	*tcp*	Thioredoxin reductase *tcpT*
	assembly_03367	*gpx1*	Hydroperoxy fatty acid reductase gpx1
	assembly_03218	*GSR*	Glutathione reductase
	assembly_00610	*OpuAA*	Glycine betaine transport ATP-binding protein *OpuAA*
	assembly_00611	*OpuAB*	Glycine betaine transport system permease protein *OpuAB*
	assembly_00612	*GbuC*	Glycine betaine/carnitine transport binding protein *GbuC*
	assembly_02651	*OpuCB*	Glycine betaine/carnitine/choline transport system permease protein *OpuCB*
	assembly_02653	*OpuCA*	Glycine betaine/carnitine/choline transport ATP-binding protein *OpuCA*
	assembly_00329		
	assembly_00354	*betA*	choline dehydrogenase *betA*
	assembly_03403		
Compatible Solute Protectants	assembly_00418		
	assembly_00839		
	assembly_01153		
	assembly_01254		
	assembly_01912		
	assembly_02035	*BetI*	transcriptional repressor *BetI*
	assembly_02139		
	assembly_02183		
	assembly_02342		
	assembly_02403		

assembly_02687		
assembly_03187		
assembly_03219		
assembly_01239	*glyA*	Serine hydroxymethyltransferase
assembly_00256	*purU*	Formyltetrahydrofolate deformylase
assembly_02002	*gltD*	Glutamate synthase [NADPH] small chain
assembly_02003	*gltB*	Glutamate synthase [NADPH] large chain
assembly_02225	*proA*	Gamma-glutamyl phosphate reductase
assembly_02226	*proB*	Glutamate 5-kinase
assembly_03301	*proC*	Pyrroline-5-carboxylate reductase
assembly_03241	*PYCR1*	1-pyrroline-5-carboxylate dehydrogenase
assembly_03135	*ProP*	Ectoine/proline transporter *ProP*
assembly_00139	*Prodh*	Proline dehydrogenase
assembly_03241	/	1-pyrroline-5-carboxylate dehydrogenase
assembly_02413	*otsA*	Trehalose-6-phosphate synthase
assembly_00956	*treS*	Maltooligosyl trehalose synthase
assembly_00957	*TreZ*	Malto-oligosyltrehalose trehalohydrolase
assembly_03018	*treP*	Alpha,alpha-trehalose phosphorylase
assembly_00029		
assembly_00116		
assembly_00582	*MalF*	Trehalose/maltose transport system permease protein *MalF*
assembly_00938		
assembly_03185		
assembly_03352		
assembly_00895	*folE*	GTP cyclohydrolase 1

Cell Membrane, Cell Wall, Extracellular Polysaccharides	assembly_01593	*folA*	Dihydrofolate reductase
	assembly_01290	*mtlD*	Mannitol-1-phosphate 5-dehydrogenase
	assembly_01291	*mtlF*	Mannitol-specific phosphotransferase enzyme IIA component
	assembly_01292	*mtlA*	PTS system mannitol-specific EIICB component
	assembly_03375	*mtlK*	Mannitol 2-dehydrogenase
	assembly_02656	*CpsY*	Exopolysaccharide phosphotransferase *CpsY*
	assembly_01281	*RfbM*	Mannose-1-phosphate guanylyltransferase *RfbM*
	assembly_01348	*manA*	Mannose-6-phosphate isomerase
	assembly_00144 assembly_02280	*YbgC/YbaW family*	acyl-CoA thioester hydrolase, *YbgC/YbaW* family
	assembly_02470	*YwqD*	Tyrosine-protein kinase *YwqD*
	assembly_01074	*ugd*	UDP-glucose 6-dehydrogenase
	assembly_01322	*YwdF*	Uncharacterized glycosyltransferase *YwdF*
	assembly_01734	*FtsW*	Probable peptidoglycan glycosyltransferase *FtsW*
	assembly_02554	*alr2836*	Uncharacterized glycosyltransferase alr2836
	assembly_03062	*mshA*	D-inositol 3-phosphate glycosyltransferase
	assembly_01075	*WbaP*	Undecaprenyl-phosphate galactose phosphotransferase
	assembly_01581	*mgtA*	GDP-mannose-dependent alpha-mannosyltransferase
	assembly_01495	*cysE*	Serine acetyltransferase
	assembly_01320	*tagU*	Polyisoprenyl-teichoic acid--peptidoglycan teichoic acid transferase *TagU*
	assembly_02178	*uppS*	Trans,polycis-polyprenyl diphosphate synthase ((2Z,6E)-farnesyl diphosphate specific)`
	assembly_02592	/	Short-chain Z-isoprenyl diphosphate synthase
	assembly_02043	/	Endolytic murein transglycosylase
	assembly_01344	*BcsY*	Putative membrane-bound transacylase *BcsY*

assembly_01529	*murE*	UDP-N-acetylmuramyl-tripeptide synthetase
assembly_01731	*murF*	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase
assembly_01732	*mraY*	Phospho-N-acetylmuramoyl-pentapeptide-transferase
assembly_01733	*murD*	UDP-N-acetylmuramoylalanine--D-glutamate ligase
assembly_01734	*FtsW*	Probable peptidoglycan glycosyltransferase *FtsW*
assembly_01735	*murG*	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase`
assembly_01736	*murC*	UDP-N-acetylmuramate--L-alanine ligase
assembly_01737	*FtsQ*	Cell division protein *FtsQ*
assembly_01738	*FtsZ*	Cell division protein *FtsZ*
assembly_01740	*SepF*	Cell division protein *SepF*
assembly_00521	*MviN*	Probable peptidoglycan biosynthesis protein *MviN*
assembly_01320	*TagU*	Polyisoprenyl-teichoic acid--peptidoglycan teichoic acid transferase *TagU*
assembly_02160	*YrhL*	Putative peptidoglycan O-acetyltransferase YrhL
assembly_00432 assembly_01717	*FadD15*	Long-chain-fatty-acid--CoA ligase *FadD15*
assembly_01662 assembly_01787	/	Delta (8)-fatty-acid desaturase
assembly_01187	*DesK*	two-component system sensor histidine kinase *DesK*
assembly_00510 assembly_01188 assembly_02673	*DesR*	Transcriptional regulatory protein *DesR*
assembly_00740	*FabG*	3-oxoacyl-[acyl-carrier-protein] reductase *FabG*
assembly_01200	*FabG*	3-oxoacyl-[acyl-carrier-protein] reductase *FabG*
assembly_	*FabZ*	3-hydroxyacyl-[acyl-carrier-protein]

01201		dehydratase *FabZ*
assembly_ 01397	*FabG*	3-oxoacyl-[acyl-carrier-protein] reductase *FabG*
assembly_ 01893	*FabG*	3-oxoacyl-[acyl-carrier-protein] reductase *FabG*
assembly_ 01960	*FabL*	Enoyl-[acyl-carrier-protein] reductase [NADPH] *FabL*

In addition, the genome contains antioxidative genes such as those encoding SOD and CAT, which are crucial for reducing oxidative damage in cold environments. This suggests that M15 has an effective antioxidative system to mitigate oxidative damage caused by cold stress, critical for the survival of both the bacterium and its rice host under low temperatures (Jeong et al., 2000; Saikolappan et al., 2011; Guan et al., 2017). Genes involved in carotenoid biosynthesis were also identified, which help scavenge ROS, thereby reducing oxidative damage induced by cold stress (Dasila et al., 2022). Genes related to the synthesis of osmoprotectants, such as betaine, trehalose, tetrahydropyrimidine, mannitol, and proline, were found to regulate intracellular osmotic pressure and protect proteins from stress-induced damage, thereby enhancing the survival rates of the bacterium and its host plant (Wemekamp-Kamphuis et al., 2004; Csonka and Leisinger, 2007; Guan et al., 2017). Furthermore, genes associated with modifications of cell membranes and cell walls, as well as those responsible for EPS synthesis and transport, were identified. These genes may help M15 resist drastic external environmental changes and enhance its survival under extreme conditions (Methé et al., 2005; Dasila et al., 2022; Kumar et al., 2022b). Notably, M15's genome contains genes responsible for EPS synthesis, indicating its ability to form biofilms that improve the rhizosphere microenvironment and enhance the plant's adaptation to cold and other stresses. Genes associated with plant growth promotion (Table 6.2), such as those involved in IAA synthesis, suggest that M15 can influence rice growth through endogenous hormone pathways (Puranik et al., 2022). Additionally, genes related to phosphate solubilization and siderophore synthesis emphasize M15's role in enhancing nutrient availability and promoting plant health (Xing et al., 2021; Silva et al., 2023; Wang et al., 2023a).

Table 6.2 Functional gene classification and annotation of genes related to plant growth promotion in the genome of *M. testaceum* M15.

PGP activities	Gene ID	Gene name	Gene annotation
indole-3-acetic acid (IAA)	assembly_ 02005	*trpA*	Tryptophan synthase alpha chain
	assembly_ 02006	*trpB*	Tryptophan synthase beta chain
	assembly_ 02007	*trpC*	Indole-3-glycerol phosphate synthase
	assembly_ 01763	*trpD*	Anthranilate phosphoribosyl transferase

	assembly_00551	*trpE*	Anthranilate synthase component 2
	assembly_01618	*trpF*	Phosphoribosylanthranilate isomerase
	assembly_01274	*trpS*	Tryptophanyl-tRNA synthetase
	assembly_00728	*amiE*	Amidase
	assembly_01963	*ywkB*	Auxin efflux carrier
	assembly_00932	*gatA*	Glutamyl-tRNA (Gln) amidotransferase subunit A
	assembly_02299	*gatA*	Glutamyl-tRNA (Gln) amidotransferase subunit A
	assembly_02298	*gatB*	Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit B
	assembly_02300	*gatC*	Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit C
	assembly_02920	*PstS*	Phosphate-binding protein
	assembly_02921	*PstC 2*	Phosphate transport system permease protein
	assembly_02922	*PstA 1*	Phosphate transport system permease protein
	assembly_02923	*PstB*	Phosphate import ATP-binding protein
	assembly_03011	*PstS*	Phosphate-binding protein
	assembly_03012	*PstA 1*	Phosphate transport system permease protein
	assembly_03013	*PstC 2*	Phosphate transport system permease protein
Phosphate solubilization	assembly_03004	*PstB*	Phosphate import ATP-binding protein
	assembly_03320	*PhoB*	Phosphate regulon transcriptional regulatory protein
	assembly_02192	*PhoH*	PhoH-like protein
	assembly_02906	*PhoU*	Phosphate transport system protein
	assembly_01040	*PhoR*	Alkaline phosphatase synthesis sensor protein
	assembly_03176	*PhnE*	Phosphate-import permease protein
	assembly_03178	*PhnC*	Phosphonates import ATP-binding protein
	assembly_03179	*PhnD2*	Probable ABC transporter phosphonate/phosphite binding protein
Iron-siderophore/Iron	assembly_	*FhuB*	Iron (3+)-hydroxamate import system

(III) transport	00298		permease protein
	assembly_ 00299	*FepG*	Ferric enterobactin transport system permease protein
	assembly_ 01884	*FepG*	Ferric enterobactin transport system permease protein
	assembly_ 01885	*FepD*	Ferric enterobactin transport system permease protein
	assembly_ 02930	*FepD*	Ferric enterobactin transport system permease protein
	assembly_ 02931	*FepG*	Ferric enterobactin transport system permease protein
	assembly_ 01571	*EfeO*	Iron uptake system component *EfeO*
	assembly_ 02390	*FhuC*	Iron (3^+)-hydroxamate import ATP-binding protein
	assembly_ 01570	*EfeU*	Ferrous iron permease
	assembly_ 01937	*VIT1*	Vacuolar iron transporter homolog 1
	assembly_ 03121	*FbpC*	Fe (3^+) ions import ATP-binding protein *FbpC*
	assembly_ 00300	*YusV*	Probable siderophore transport system ATP-binding protein
	assembly_ 02929	*YfiY*	Probable siderophore-binding lipoprotein *YfiY*
	assembly_ 02390	*FhuC*	Iron (3^+)-hydroxamate import ATP-binding protein FhuC
	assembly_ 02993	/	siderophore-interacting protein
	assembly_ 01880	*YitW*	Fe-S protein maturation auxiliary factor
	assembly_ 01277	/	Succinate dehydrogenase iron-sulfur subunit
	assembly_ 01760	/	Cytochrome bc1 complex Rieske iron-sulfur subunit
	assembly_ 02028	/	Putative Rieske 2Fe-2S iron-sulfur protein
	assembly_ 02507	/	Iron-sulfur cluster carrier protein
	assembly_ 01402	/	Flagellin
	assembly_ 01403	/	Flagellar filament capping protein
Colonization	assembly_ 01404	*FliS*	Flagellar export chaperone
	assembly_ 01406	*FlgB*	Fagellar biosynthesis protein
	assembly_ 01407	*FlgC*	Flagellar basal body rod protein
	assembly_	*FliE*	Flagellar hook-basal body complex

01408		protein
assembly_01409	*FliF*	Flagellar M-ring protein
assembly_01410	*FliG*	Flagellar motor switch protein
assembly_01411	*FliH*	Flagellar assembly protein
assembly_01412	*FliI/YscN family ATPase*	Flagellum-specific ATP synthase
assembly_01413	*FliJ*	Flagellar *FliJ* protein
assembly_01414	*FliK*	Flagellar hook-length control protein
assembly_01415	*FlgD*	Flagellar hook capping protein
assembly_01416	*FlgE*	Flagellar hook protein
assembly_01417	*FlbD*	Flagellar protein
assembly_01418	*MotA*	Motility protein A
assembly_01419	*MotB*	Flagellar motor protein
assembly_01420	*FliM*	Flagellar motor switch protein
assembly_01421	*FliN*	Flagellar motor switch protein
assembly_01422	*FliO*	Flagellar biosynthesis protein
assembly_01423	*FliP*	Flagellar biosynthetic protein
assembly_01424	*FliQ*	Flagellar biosynthetic protein
assembly_01425	*FliR*	Flagellar biosynthetic protein
assembly_01426	*FlhB*	Flagellar biosynthetic protein
assembly_01427	*FlhA*	Flagellar biosynthesis protein
assembly_01429	*FlgN*	Flagellar protein
assembly_01430	*FlgK*	Flagellar hook-associated protein
assembly_01431	*FlgL*	Flagellar hook-associated protein 3
assembly_01432	*FliW*	Flagellar assembly protein

GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), and COG pathway analyses further revealed that M15's metabolic pathways involve cell communication, material transport, stress responses, carbohydrate and amino acid metabolism, membrane transport, and energy conversion (Figures 6.2-6.4). These results highlight M15's multifunctionality under extreme conditions and its ability to regulate plant physiological processes to enhance growth and cold tolerance. M15 promotes rice growth not only through direct biological interactions but also by regulating metabolic pathways that support plant responses to cold, thereby enhancing physiological adaptation.

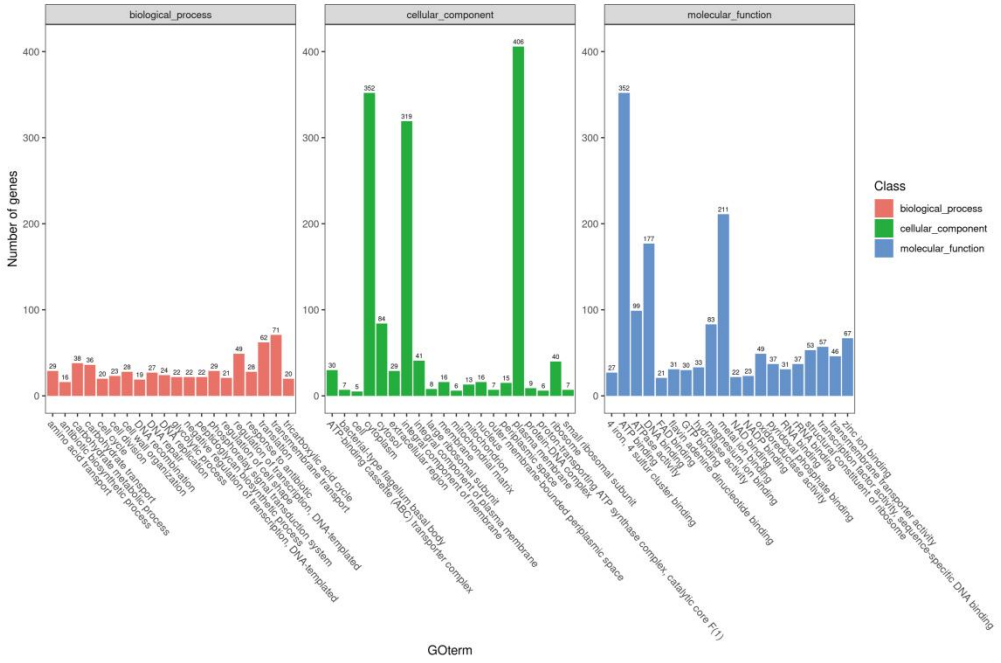

Figure 6.2 Gene Ontology (GO) analysis of M15 strain. This figure illustrates the gene enrichment of the M15 strain across the three main categories of Gene Ontology: biological processes, molecular functions, and cellular components.

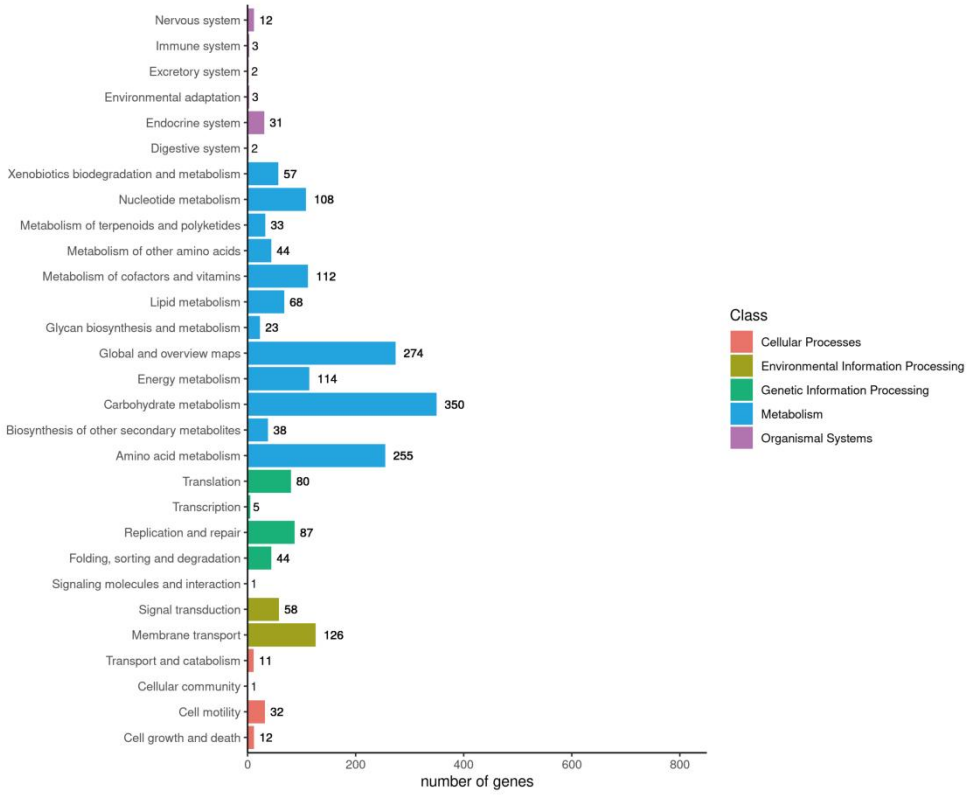

Figure 6.3 KEGG pathway analysis of the M15 strain. This figure presents the results of KEGG pathway analysis, identifying key biological metabolic pathways associated with the M15 strain, including cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems.

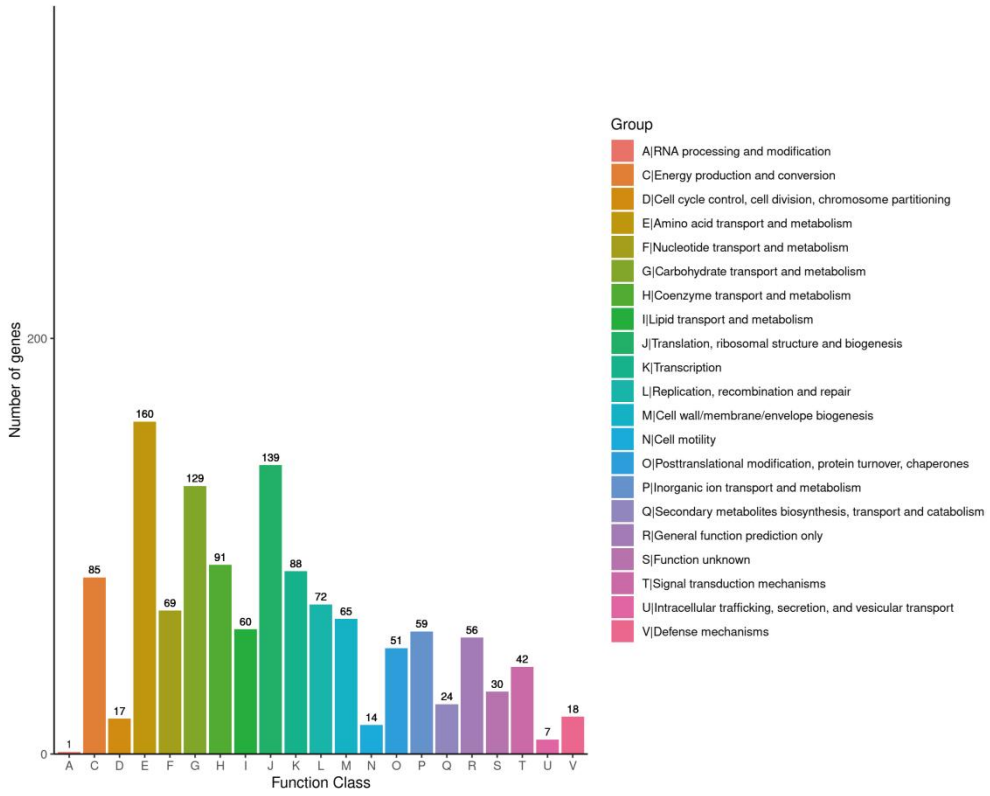

Figure 6.4 COG functional classification of the M15 strain. This figure depicts the functional classification of genes within the M15 genome based on the COG database. It highlights the significant enrichment of genes in functional categories related to amino acid transport and metabolism, carbohydrate transport and metabolism, translation, ribosome structure and biogenesis, coenzyme metabolism, transcription, and cell cycle control and division.

In summary, genomic analysis demonstrates that M15 possesses robust cold adaptation capabilities and can promote rice growth and cold tolerance through multiple mechanisms (Figure 6.5). These findings underscore the potential of endophytic bacteria to enhance crop performance, laying the foundation for applications in agricultural biotechnology (Kushwaha et al., 2020). M15 shows considerable promise as a microbial inoculant for improving crop resilience to environmental stresses.

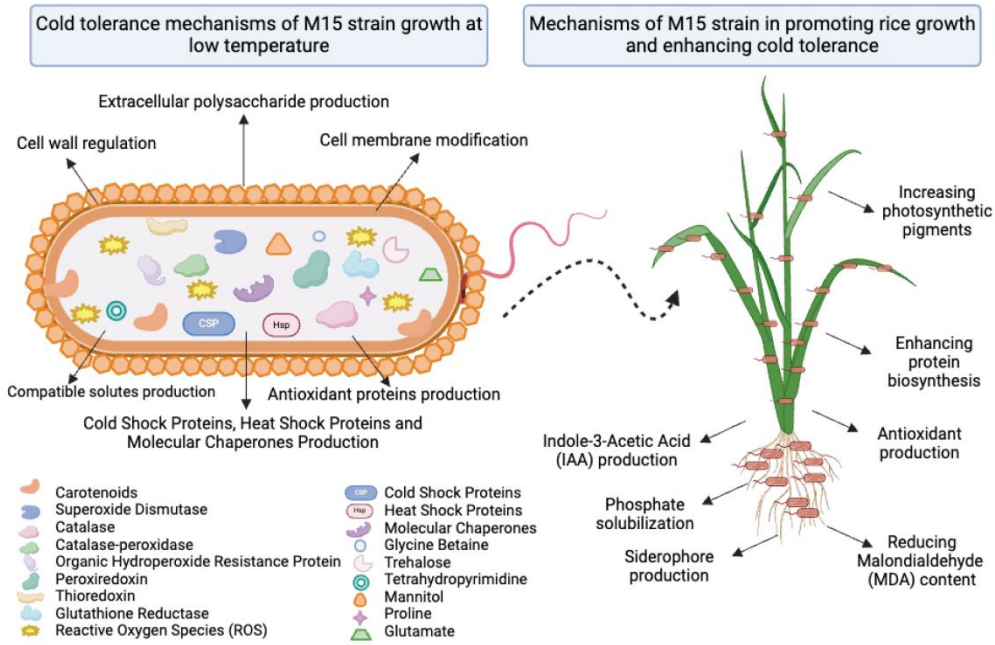

Figure 6.5 Mechanisms of M15 in enhancing cold tolerance and promoting growth in rice under cold stress. The cold tolerance mechanisms of M15 include the production of cold shock proteins, heat shock proteins, and molecular chaperones; regulation of cell wall properties; modification of cell membranes; synthesis of compatible solute protectants; and production of antioxidant proteins to mitigate oxidative damage caused by cold stress. The mechanisms by which M15 promotes growth and enhances cold tolerance in rice include the production of indole-3-acetic acid (IAA); phosphate solubilization; siderophore production; increased levels of photosynthetic pigments; enhanced protein biosynthesis; antioxidant production; and reduced malondialdehyde (MDA) content.

6.3.1.2 Transcriptomic analysis of M15

To further elucidate the changes in gene expression in *Microbacterium testaceum* M15 under cold stress, we conducted a comparative transcriptomic analysis of the strain grown under normal (37℃) and cold (4℃) conditions. Differentially expressed genes (DEGs) were identified, and KEGG pathway analysis revealed that multiple genes associated with the phosphate regulatory system (Pho system) and the phosphate-specific transport system (Pst system) were significantly upregulated under cold conditions (Figures 6.6A and B). Detailed functions of these phosphate transport genes are provided in Table 6.3. The upregulation of these genes under cold stress suggests that M15 activates its phosphate metabolism pathways in response to low temperatures, particularly genes involved in phosphate transport. This highlights the critical role of phosphate transport genes in enhancing the cold tolerance of M15 and subsequently promoting phosphate absorption and stress tolerance in rice. By improving

phosphate bioavailability, M15 enhances plant growth and adapts to cold environments through optimized phosphate metabolism mechanisms.

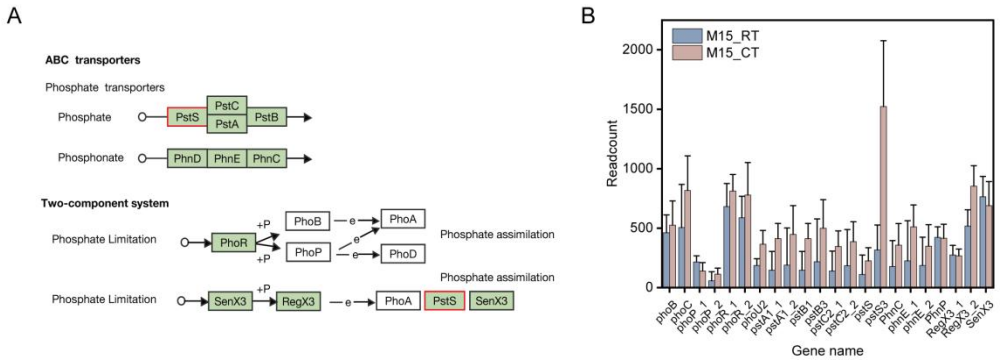

Figure 6.6 Transcriptomic pathway analysis and gene expression profiling of phosphate transport in *M. testaceum* M15 under different temperature conditions. (A) KEGG pathway analysis of phosphate transport genes in *M. testaceum* M15 under different temperature conditions. The transcriptional profiles of phosphate transport-related genes were analyzed at normal temperature (37℃) and low temperature (4℃). Genes actively involved in the phosphate transport pathway are highlighted in green boxes. Genes present in the pathway but with no significant expression changes are shown on a white background. Upregulated genes are marked in red. (B) Expression levels of genes in the phosphate transport systems (Pho and Pst) in M15 at normal temperature (37℃) and low temperature (4℃). M15_RT represents *M. testaceum* M15 cultured at 37℃, while M15_CT represents *M. testaceum* M15 cultured at 4℃. Data were derived from three independent biological replicates.

Table 6.3 List of genes related to phosphate metabolism identified in the transcriptomic analysis of M15.

Category	Gene ID	Gene name	Product
Phosphate Transport	assembly_02914	*phoB*	Phosphate regulon transcriptional regulatory protein *PhoB*
	assembly_01533	*phoC*	Major phosphate-irrepressible acid phosphatase
	assembly_00180	*phoP_1*	Alkaline phosphatase synthesis transcriptional regulatory protein *PhoP*
	assembly_00783	*phoP_2*	Alkaline phosphatase synthesis transcriptional regulatory protein *PhoP*
	assembly_01040	*phoR_1*	Alkaline phosphatase synthesis sensor protein *PhoR*
	assembly_01842	*phoR_2*	Alkaline phosphatase synthesis sensor protein *PhoR*
	assembly_02906	*phoU2*	Phosphate-specific transport system accessory protein *PhoU*

assembly_02922	*pstA1_1*	Phosphate transport system permease protein *PstA1*
assembly_03012	*pstA1_2*	Phosphate transport system permease protein *PstA1*
assembly_02923	*pstB1*	Phosphate import ATP-binding protein *PstB1*
assembly_03004	*pstB3*	Phosphate import ATP-binding protein *PstB3*
assembly_02921	*pstC2_1*	Phosphate transport system permease protein *PstC2*
assembly_03013	*pstC2_2*	Phosphate transport system permease protein *PstC2*
assembly_03011	*pstS*	Phosphate-binding protein *PstS*

The upregulation of genes in the Pho and Pst systems under cold stress underscores their role in increasing the strain's capacity for phosphate uptake and utilization from the environment. Phosphate is not only vital for microbial growth but also provides essential nutrients for plants. As a crucial nutrient, phosphate plays a key role in plant cell signaling, energy metabolism, and cell membrane composition, especially under environmental stress (Malhotra et al., 2018). The activation of phosphate metabolism in M15 under cold conditions supports the survival of the strain and may also enhance phosphate use efficiency in rice, thereby improving cold tolerance.

By enhancing phosphate utilization efficiency and optimizing plant phosphate uptake, M15 may provide essential physiological support to rice, helping the host plant maintain higher growth rates and cold tolerance under cold conditions. While cold stress typically limits phosphate uptake in plants, it simultaneously increases the demand for phosphate. M15 addresses this challenge by boosting phosphate transport and uptake, ensuring sufficient phosphate availability for rice and thereby promoting growth and cold tolerance. Studies have shown that under cold stress, microorganisms can enhance plants' stress tolerance by increasing phosphate uptake and transport capabilities (Rizvi et al., 2021). For instance, the cold-tolerant plant growth-promoting *Pseudomonas* strain PGERs17 (MTCC 9000), isolated from the Northwestern Himalayas of India, exhibited phosphate solubilization activity and improved wheat seedling germination rates, root length, and shoot length under cold conditions (Mishra et al., 2008). Similarly, cold-tolerant bacteria isolated from wild plants in the Andes and Patagonia in Chile displayed phosphate solubilization traits that promoted plant growth and alleviated cold stress (Vega-Celedón et al., 2021).

Through the upregulation of phosphate transport-related genes and optimization of its phosphate metabolism pathways, M15 not only promotes its own growth under cold stress but also provides the host plant with the phosphate required for survival and adaptation. This improves the phosphate use efficiency of rice and enhances its cold tolerance. These findings deepen our understanding of the cold tolerance mechanisms of M15 and offer new perspectives for utilizing microorganisms to

enhance crop resilience to cold stress. They highlight the critical role of phosphate metabolism and transport in this process, providing valuable insights into the mechanisms by which M15 facilitates cold tolerance in rice under cold conditions.

6.3.2 Enhancement of phosphorus utilization and cold tolerance in rice by M15

The genomic and transcriptomic analyses of the M15 strain highlighted its functions related to cold tolerance and plant growth promotion. The application effects on rice further confirmed M15's potential to enhance cold tolerance and growth. To investigate the role of phosphorus solubilization by M15 in improving rice cold tolerance, the solubilization capacity of M15 and its effects on rice cold tolerance were comprehensively evaluated.

6.3.2.1 Phosphorus solubilization analysis of M15

The M15 strain exhibited significant phosphorus solubilization ability in NBRIP medium. By measuring pH changes in the medium, it was observed that M15 significantly reduced the pH from 7.97 ± 0.10 to 4.95 ± 0.01 within 24 hours (Figure 6.7A). This reduction suggests that M15 enhances the solubility of phosphorus in the environment through acidification, facilitating its effective release. Additionally, gluconic acid, a key solubilizing agent for phosphate, was detected in the medium inoculated with M15, indicating its crucial role in the phosphorus solubilization mechanism of M15 (Figure 6.7B). Gluconic acid, an important organic acid, effectively dissolves inorganic phosphorus and enhances its bioavailability, providing adequate phosphorus sources for rice (Mei et al., 2021).

These results demonstrate that M15 improves phosphorus solubility by lowering pH and secreting gluconic acid, thereby increasing the availability of phosphorus in the environment. This is critical for plant growth under stress conditions. Previous studies reported that gluconic acid is a major organic acid involved in phosphate solubilization by JP233 bacteria, and the application of phosphate-solubilizing bacteria (PSB) significantly reduces phosphate leaching losses while promoting the absorption and utilization of soil phosphorus by plants (Yu et al., 2022).

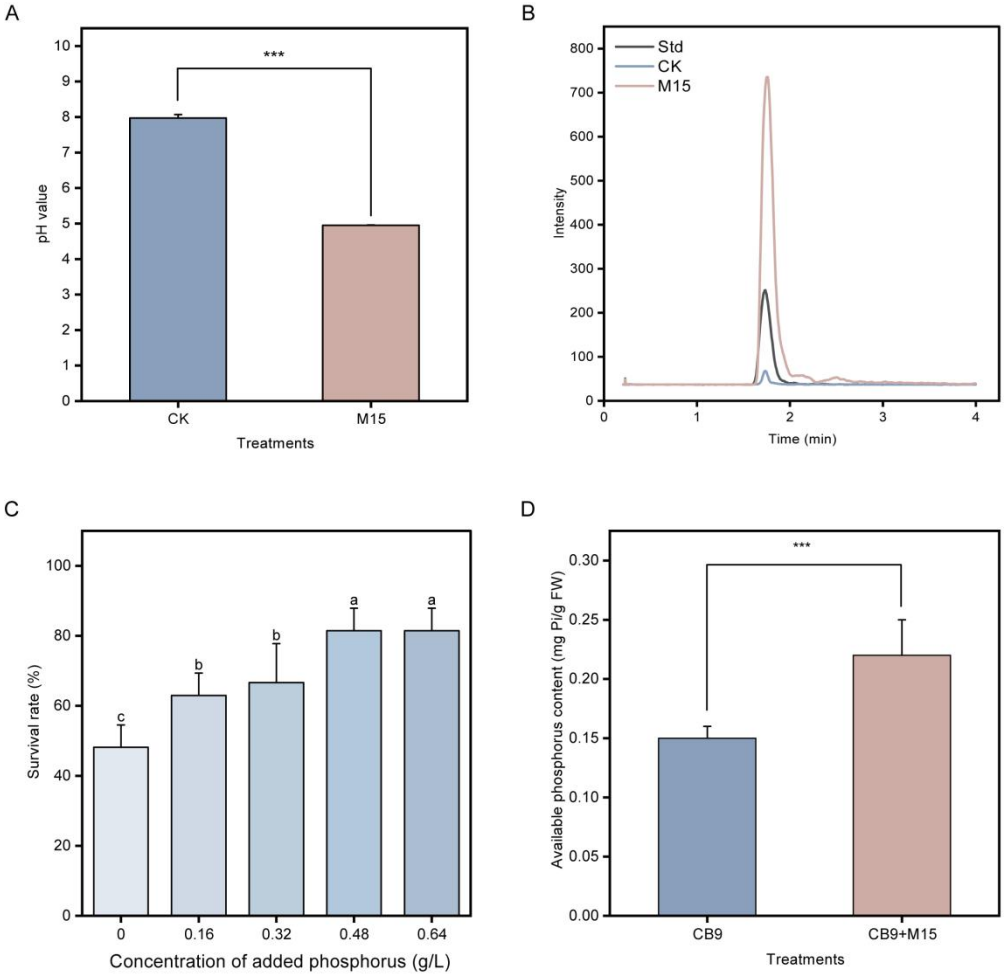

Figure 6.7 Phosphate solubilization by M15 and its role in enhancing rice cold tolerance. (A) pH values of NBRIP medium after 1 day of culture with and without M15 inoculation. (B) Gluconic acid content in the supernatant of NBRIP medium after 1 day of culture with and without M15 inoculation, measured by LC-QQQ. (C) Survival rates of rice seedlings under different concentrations of exogenous phosphate after cold stress (4℃) followed by recovery at normal temperature (26℃). (D) Bioavailable phosphate content in rice seedling leaves after M15 inoculation. CK refers to NBRIP medium without M15 inoculation; M15 refers to NBRIP medium with M15 inoculation; STD refers to the standard gluconic acid; CB9 refers to seedlings without M15 inoculation; CB9+M15 refers to seedlings inoculated with M15. Data are presented from three independent biological replicates. Means with the same letters are not significantly different at $p < 0.05$.

The phosphorus solubilization capacity of M15 ensures sufficient phosphorus supply for rice, especially under cold stress. Effective phosphorus utilization

promotes energy metabolism and growth in plants, helping maintain physiological balance and tolerance under adverse conditions. This finding further supports the role of M15 in enhancing rice adaptation to cold stress through its phosphorus solubilization ability.

6.3.2.2 Effects of exogenous phosphorus on rice cold tolerance

To validate whether exogenous phosphorus enhances rice cold tolerance, different concentrations of phosphorus were added to the rice culture medium, and the survival rates under cold stress were evaluated. Results showed that the addition of 0.64 g/L phosphorus during the rice seedling stage significantly increased survival rates from 48.15 ± 6.42% in the control group to 81.48 ± 6.42% (Figure 6.7C). This indicates that exogenous phosphorus effectively enhances rice tolerance to cold stress, further confirming the critical role of phosphorus in plant cold tolerance.

Phosphorus is an essential nutrient for plants, playing vital roles in cellular processes such as energy metabolism, signal transduction, and photosynthesis, as well as being a major component of phospholipids, ATP, and nucleic acids (Vance et al., 2003). Studies have reported that soil supplemented with P fertilizer and phosphate-solubilizing bacteria (P + B+) significantly reduces cellular damage (as indicated by lipid peroxidation) and increases the expression of Pi transporter and SOD isozyme genes, which are markers of improved stress tolerance in plants (Barra et al., 2019).

Under cold conditions, the demand for phosphorus in rice increases. Supplementing exogenous phosphorus improves energy metabolism and physiological functions during cold stress, enhancing rice adaptation to cold environments. These experimental results align with the findings that M15 enhances rice phosphorus utilization through solubilization, further demonstrating its potential in improving rice cold tolerance. By boosting phosphorus absorption and utilization, M15 enhances rice adaptability under cold conditions.

6.3.2.3 Effects of M15 on phosphorus content in rice

Further analysis was conducted to evaluate the effects of M15 on phosphorus content in rice. Results showed that inoculation with M15 significantly increased the effective phosphorus content in rice leaves from 0.15 ± 0.01 mg Pi/g FW in the control group to 0.22 ± 0.03 mg Pi/g FW (Figure 6.7D). This increase may be attributed to the phosphorus solubilization effect of M15, which enhances the availability of phosphorus in the environment and consequently improves its absorption and utilization by rice.

Increased effective phosphorus content is crucial for rice growth under cold conditions. Adequate phosphorus supply improves plant energy metabolism and ATP synthesis, providing sufficient energy for physiological activities under cold stress. Enhanced phosphorus content supplies rice with more energy and signaling molecules, improving its physiological activities under low temperatures. This helps maintain physiological balance and promotes growth and activity, ultimately strengthening rice cold tolerance.

Previous studies have shown that microbial inoculation can enhance effective

phosphorus content in plants. For instance, inoculation with *Streptomyces roseocinereus* MS1B15 significantly increased the lengths of shoots and spikes, as well as the effective phosphorus content in spikes and leaves, along with P and N content in the soil (Chouyia et al., 2020). Similarly, PSB inoculation significantly improved shoot biomass, enhanced physiological functions in roots and shoots, and increased total phosphorus in shoots and root phosphatase activity (Elhaissoufi et al., 2020). PSMs not only increase phosphorus content in plants but also improve overall growth and health under both normal and stress conditions (Rawat et al., 2021). Therefore, by enhancing phosphorus solubilization, M15 increases effective phosphorus content in rice, improving its adaptation and tolerance to cold conditions.

6.4 Conclusion

This study comprehensively investigated the genomic, transcriptomic, and functional characteristics of *M. testaceum* M15, revealing its potential for enhancing phosphorus utilization and cold tolerance in rice. The complete genome sequencing of M15 uncovered an array of genes associated with environmental stress adaptation, including those involved in CSPs, HSPs, antioxidant enzymes such as SOD and CAT, osmotic protectants like betaine and trehalose, and EPS synthesis. These genetic elements equip M15 with robust environmental adaptability and the capacity to alleviate abiotic stresses in plants. Furthermore, genes associated with phosphorus solubilization, auxin synthesis, and siderophore production indicate the strain's ability to enhance nutrient availability and promote plant growth.

Transcriptomic analysis under cold stress conditions revealed significant upregulation of genes related to the Pho and Pst systems, which are critical for phosphate regulation and transport. These findings suggest that M15 activates its phosphorus metabolic pathways in response to low temperatures, enhancing the bioavailability of phosphorus. This capability underscores its role in facilitating the adaptation of rice to cold environments, particularly by optimizing nutrient acquisition under stress conditions.

The functional assessment of M15 demonstrated its notable ability to solubilize inorganic phosphate, as evidenced by a significant reduction in the pH of the NBRIP medium and the production of gluconic acid. This key organic acid plays a vital role in increasing phosphorus solubility, thereby improving its availability for plant uptake. Additionally, exogenous phosphorus supplementation experiments confirmed the critical role of phosphorus in plant cold tolerance. These experiments showed that phosphorus not only supports energy metabolism and physiological balance but also enhances rice survival under cold stress. Inoculation with M15 significantly increased the effective phosphorus content in rice leaves, further corroborating its role in facilitating nutrient acquisition and stress resilience.

Overall, this study highlights the multifunctional potential of *M. testaceum* M15 as a microbial inoculant for improving rice growth and cold tolerance. By leveraging its genomic and transcriptomic adaptations and functional attributes, M15 enhances rice productivity under challenging environmental conditions. These findings

underscore the importance of utilizing microbial-based solutions for sustainable agriculture, particularly in mitigating the adverse impacts of abiotic stresses. The research provides a solid foundation for further exploration of M15's applications in improving crop resilience and advancing environmentally sustainable farming practices.

Chapter 7

Evaluation of the application potential of *Microbacterium testaceum* M15 in rice cultivation under cold conditions

Shifting focus from the molecular mechanisms explored in Chapter 6, this chapter evaluates the practical application potential of *M. testaceum* M15 in rice cultivation under cold stress conditions. The effects of *M. testaceum* M15 on the cold tolerance and agronomic traits of CB9 rice during the reproductive stage are assessed, along with its regulatory role on the diversity and composition of grain-associated microbial communities. This chapter bridges laboratory research with real-world agricultural applications, offering practical insights into the use of *M. testaceum* M15 in farming.

7.1 Introduction

Cold stress is one of the major abiotic factors that affect rice growth and yield. In addition to the cold stress that rice seedlings face, cold stress during the booting stage also severely impacts rice growth and yield (Shi et al., 2024b). The booting stage is a critical phase in the reproductive growth of rice, directly affecting later panicle differentiation, pollen development, and grain filling. cold stress not only inhibits rice's vegetative growth and reduces photosynthetic efficiency but also hinders panicle differentiation and development. In severe cases, it causes poor grain filling, lower seed-setting rates, reduced 1,000-grain weight, and ultimately results in higher empty grain rates and a significant yield decrease (Ghadirnezhad and Fallah, 2014; Gao et al., 2024). Additionally, low temperatures induce a large accumulation of ROS in plant cells, causing lipid peroxidation in cell membranes, severely damaging cell structures and functions, and leading to metabolic disorders (Sachdev et al., 2021). Therefore, alleviating the damage caused by low temperatures during the booting stage of rice and improving its cold tolerance is crucial for increasing rice yield and quality.

The development and utilization of microbial resources, especially endophytic bacteria with growth-promoting and stress-resistant properties, have become effective strategies to enhance crop stress tolerance and productivity. Although research on how microorganisms improve rice cold tolerance during the booting stage is still in its infancy, studies have demonstrated the significant potential of plant growth-promoting microorganisms in enhancing plant cold tolerance. Microorganisms enhance plants' ability to adapt to low temperatures through various mechanisms, such as improving antioxidant capacity, regulating plant hormone levels, and modulating rhizosphere microbial communities.

The colonization ability of microorganisms is the foundation for their growth-promoting and stress-tolerance functions. Studies have found that factors such as bacterial surface characteristics, EPS secretion ability, and biofilm formation ability play key roles in their colonization in plants (Wang et al., 2023c). The secretion of EPS helps bacteria attach to the roots of plants and form biofilms, thereby enhancing their stability and functionality within the plant. After microbial inoculation via foliar spraying or root irrigation, microorganisms use surface features such as adhesion proteins and outer membrane proteins to bind to the plant tissue surface, forming tight attachments. Subsequently, microorganisms proliferate and form stable microbial communities in the plant environment. Microorganisms can also attach to the seed surface or enter the plant embryo during seed germination, becoming endophytic microorganisms. These microbes can maintain a symbiotic relationship with the host plant throughout its life cycle, thus influencing the health, stress tolerance, and growth of the offspring (Berg and Raaijmakers, 2018). Additionally, the mother plant passes the microorganisms to the next generation through seeds or pollen, enabling intergenerational transmission (Frank et al., 2017). The colonization of exogenous microorganisms not only helps plants adapt to adverse environments through direct ecological interactions but may also improve the stress tolerance of offspring plants by transferring them to future generations.

Exogenous microbial inoculation can not only colonize within the plant but also regulate the microbial community structure of the host plant, enhancing the plant's adaptability to environmental stresses. Studies have shown that the diversity and stability of plant endophytic microbial communities are closely related to plant health and stress tolerance (Afzal et al., 2019). Microorganisms regulate the endophytic microbial community by competitive exclusion, resource competition, and secretion of antimicrobial substances, reducing potential pathogens and increasing beneficial microbes (Hassani et al., 2018). The optimization of this microecological balance plays an important role in enhancing plant growth and stress tolerance.

Microbacterium testaceum M15 is an endophytic bacterium with plant growth-promoting effects. Previous research has shown that M15 can significantly promote rice growth and enhance the cold tolerance of rice seedlings. However, the cold tolerance effect of M15 during the booting stage and its impact on the endophytic microbiome in mature grains remain unclear. Therefore, exploring the mechanisms of M15 in rice during the booting stage is of great theoretical value and application potential.

This study aims to systematically investigate the cold tolerance effects of M15 on the cold-sensitive rice variety CB9 under cold stress during the booting stage, and to analyze in depth the regulatory effects of M15 inoculation on rice physiological metabolism, antioxidant systems, key agronomic traits, and the structure of the endophytic microbial community in mature grains. The specific research content includes: evaluating the impact of M15 on rice cold tolerance during the booting stage by measuring the MDA content and SOD activity in rice leaves. Assessing the comprehensive effects of M15 on rice growth and yield by analyzing agronomic traits such as plant height, panicle length, 1,000-grain weight, number of filled grains, and number of unfilled grains. Investigating the effect of M15 inoculation on the structure and diversity of the endophytic microbial community in mature rice grains through high-throughput sequencing, exploring the role of M15 in regulating the endophytic microbial community and its influence on plant microecology. This study will reveal the mechanisms of M15 in enhancing rice cold tolerance during the booting stage, improving crop growth, and regulating the endophytic microbial community. The findings will contribute to a deeper understanding of M15's role in promoting rice growth and enhancing cold tolerance during the booting stage, providing scientific evidence for elucidating its growth-promoting and cold tolerance mechanisms in rice during this critical phase.

7.2 Materials and methods

7.2.1 Pot experiments throughout the rice growth cycle

To investigate the effect of *M. testaceum* M15 on the cold tolerance of the cold-sensitive rice variety CB9 during the booting stage, this study established two groups: a control group (CB9) and an experimental group (CB9+M15). Both groups underwent systematic treatment starting from seed soaking, transplantation, root

immersion, and subsequent root drenching with bacterial suspension to comprehensively evaluate the influence of M15 on rice growth and cold tolerance.

7.2.1.1 Seed soaking and root immersion treatments

Prior to sowing, seeds were subjected to soaking treatments. Seeds in the control group were soaked in sterile water, while those in the experimental group were treated with M15 suspension at a concentration of 1×10^8 CFU/mL, ensuring sufficient absorption of functional bacterial components. After soaking, rice seedlings were cultivated to the three-leaf one-heart stage, and uniformly grown healthy seedlings were selected for transplantation.

During transplantation, seedlings from each group underwent root immersion treatment. Roots of the control group were immersed in sterile water, while those of the experimental group were immersed in M15 suspension. Specifically, seedling roots were completely submerged in the respective treatment solution for six hours to ensure uniform coating of M15 suspension on the root surface.

7.2.1.2 Regular root drenching with bacterial suspension

After transplantation, seedlings were planted in standard potting setups and managed under conventional cultivation conditions. Throughout the growth period, root drenching was performed monthly. For the control group, sterile water was used, while an equivalent volume of M15 suspension (1×10^8 CFU/mL) mixed with sterile water was applied to the experimental group. The root drenching ensured the sustained influence of M15 on the rhizosphere microbial community and rice growth.

7.2.1.3 Cold stress treatment and physiological index measurements

At the booting stage, rice plants underwent cold stress treatment. All groups were subjected to cold stress at 14℃ for 72 hours, followed by recovery under standard cultivation conditions for 72 hours. This process aimed to evaluate the impact of cold stress on rice physiological status and recovery capacity.

To explore the physiological effects of cold stress, the last leaf (LL), second leaf (SL), and third leaf from the top (TTL) were selected for measurement of two key physiological indicators:

MDA (Malondialdehyde) Content: An indicator of membrane lipid peroxidation, reflecting cell membrane damage under cold stress.

SOD (Superoxide Dismutase) Activity: A key enzyme in the antioxidant system, indicating the plant's ability to mitigate oxidative stress caused by cold damage.

Measurement of MDA Content:

MDA content was determined using the thiobarbituric acid (TBA) reaction, where the MDA-TBA complex exhibits a characteristic absorption peak at 532 nm.

Sample preparation: A 0.5 g leaf sample (LL, SL, TTL) was ground with 5 mL of 10% (w/v) trichloroacetic acid (TCA) solution in a pre-cooled mortar. The homogenate was centrifuged at 12,000 rpm at 4℃ for 10 minutes, and the supernatant was collected for measurement.

Reaction procedure: A 2 mL supernatant was mixed with an equal volume of 0.6% (w/v) TBA solution in 10% TCA. The mixture was heated in a boiling water bath for 15 minutes and then cooled rapidly to room temperature.

Absorbance Measurement: After centrifugation, the supernatant's absorbance was measured at 532 nm and 600 nm using a spectrophotometer. MDA content was calculated using the formula: MDA ($nmol \cdot g^{-1}$ FW)=6.45 × (OD_{532}−OD_{600}) / fresh weight.

Measurement of SOD activity:

SOD activity was measured using the nitroblue tetrazolium (NBT) photochemical reduction method. SOD inhibits NBT reduction, decreasing the formation of a blue compound.

Sample preparation: A 0.5 g leaf sample (LL, SL, TTL) was homogenized with 5 mL of pre-cooled phosphate buffer (0.05 M, pH 7.8). The homogenate was centrifuged at 12,000 rpm for 10 minutes, and the supernatant served as the crude enzyme extract.

Reaction mixture: A total reaction volume of 3 mL was prepared, consisting of: 1.5 mL 50 mM phosphate buffer (containing 1 mM EDTA), 0.3 mL 130 mM NBT, 0.2 mL 50 mM L-methionine, 0.3 mL 0.1 mM riboflavin, 0.1 mL enzyme extract. The reaction was conducted under light for 20 minutes, with a dark control as a negative control.

Absorbance measurement: Absorbance was measured at 560 nm. SOD activity was calculated as: SOD ($U \cdot g^{-1}$ FW)=(Control Absorbance−Sample Absorbance) / Control Absorbance × 50% × Dilution Factor

7.2.1.4 Agronomic trait measurement at maturity

After completing cold stress treatment and recovery, rice plants were cultivated to maturity, and the following key agronomic traits were measured to evaluate the comprehensive impact of M15:

Plant height: The vertical height from the root collar to the top of the main panicle.

Panicle length: The length of the longest panicle per plant.

Number of panicles: The number of tillers and panicles per plant.

Thousand-grain weight: The average weight of 1,000 randomly selected grains.

Filled grain weight: The total weight of mature grains per plant.

Unfilled grain weight: The total weight of unripe or empty grains per plant.

7.2.1.5 Statistical analysis

All experiments were performed with three independent biological replicates, and the results are expressed as mean ± standard error (mean ± SE). For comparisons between two groups, an independent sample t-test was conducted. Statistical analyses were performed using IBM SPSS Statistics 20 software (IBM Corp., Armonk, NY, USA). The significance level was set at $p < 0.05$. In the figures, “ns” indicates no significant difference ($p > 0.05$); “*” indicates significant difference ($p < 0.05$); and “**” indicates highly significant difference ($p < 0.01$). All graphical

presentations were generated using Origin Pro 2021 software (OriginLab Corporation, USA).

7.2.2 Analysis of endophytic microbial diversity in mature rice grains

To verify whether *M. testaceum* M15 could successfully colonize CB9 rice and to investigate its effects on the composition of the microbial community in mature rice grains, the endophytic microbial diversity of CB9 control and CB9+M15 treatment groups was analyzed.

7.2.2.1 Sample preparation and DNA extraction

To further explore the regulatory effects of M15 on the endophytic microbial community of rice, mature rice grains (varieties JG117 and CB9) were selected for diversity analysis. Grains were randomly collected from each treatment group. After removing surface debris, 2 g of sample was weighed. The samples were immersed in 75% ethanol for 10 minutes, followed by rinsing with sterile distilled water five times. They were then treated with a 10% sodium hypochlorite solution for 10 minutes and rinsed three more times with sterile distilled water to ensure complete removal of surface microorganisms.

The sterilized grains were placed in a sterile mortar and ground into a fine powder using a sterile pestle. A portion of the fine powder was used for genomic DNA extraction with a commercial DNA extraction kit. The quality of the extracted DNA was assessed via agarose gel electrophoresis.

7.2.2.2 16S rRNA gene amplification and sequencing

Genomic DNA samples were amplified using universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the V3–V4 region of the 16S rRNA gene. Amplification products were purified by agarose gel electrophoresis and subsequently sequenced using the Illumina MiSeq platform with the PE250 paired-end sequencing strategy. Sequencing data processing and analysis were performed by Beijing Allwegene Gene Technology Co., Ltd.

7.2.2.3 Data analysis and microbial diversity assessment

The raw sequencing data were processed using FastQC (Babraham Bioinformatics, UK) for quality control, and Trimmomatic (USA) was employed for read trimming. High-quality reads were merged using Pear (v0.9.6) and chimera sequences were removed with Vsearch (v2.7.1) using the uchime algorithm. Operational taxonomic units (OTUs) were clustered at a 97% similarity threshold using the uparse algorithm in Vsearch. OTU classification was performed using BLAST against the Silva 138 database with an e-value threshold of 1e-5.

Microbial diversity analysis including:

Alpha diversity: Community richness was assessed using the Chao index and Observed Species index. Community diversity was evaluated using the Shannon and Simpson indices.

Beta diversity: Community similarity between samples was calculated using Bray-Curtis and Weighted UniFrac distance matrices. Community composition differences were visualized using:

Non-metric multidimensional scaling (NMDS), Principal coordinates analysis (PCoA), Partial least squares discrimination analysis (PLS-DA), and Principal component analysis (PCA).

Taxonomic annotation and composition analysis: The primary microbial taxa and their relative abundances in different treatment groups were analyzed. Bar plots illustrated the distribution differences of dominant microbial taxa between groups. At the genus and species levels, relatively abundant taxa and their distribution differences were analyzed. Heatmaps were generated to present significantly enriched taxa based on species abundance clustering.

Statistical analysis - wilcoxon test: Wilcoxon rank-sum test was used to analyze differences in microbial relative abundances between treatment groups.

LEfSe (Linear Discriminant Analysis Effect Size): LEfSe was used to identify biomarkers, i.e., taxa with significantly different abundances between groups. Linear discriminant analysis (LDA) scores greater than 3 and p-values less than 0.05 were considered significant.

7.3 Results and discussion

7.3.1 The effects of M. testaceum M15 on the cold tolerance of CB9 rice during the booting stage

The booting stage represents another critical period of rice growth and development, characterized by more complex physiological requirements and environmental adaptability compared to the seedling stage. During this stage, cold stress not only impairs rice growth but also causes poor panicle differentiation, reduced seed-setting rates, and significant declines in yield and quality (Ghadirnezhad and Fallah, 2014; Kang et al., 2022; Gao et al., 2024). This study extended the investigation from the seedling stage to the booting stage to assess the effects of *M. testaceum* M15 under cold stress conditions. The study focused on improving physiological parameters such as MDA content and SOD activity to evaluate the role of M15 in enhancing cold tolerance in CB9 rice during the booting stage.

7.3.1.1 Physiological parameter changes in rice after under stress at the booting stage

Rice plants at the booting stage were subjected to cold stress at 14℃ for 72 hours, followed by a recovery period of 72 hours. The MDA content, an indicator of lipid peroxidation, and SOD activity, a critical antioxidant enzyme, were measured in three leaf positions: the last leaf (LL), the second leaf (SL), and the top third leaf (TTL). Results showed that cold stress significantly increased MDA content in the CB9 control group (without M15 inoculation), with levels recorded as 14.00 ± 1.10 nmol/g, 20.52 ± 5.25 nmol/g, and 32.69 ± 3.53 nmol/g for LL, SL, and TTL,

respectively (Figure 7.1A). This indicates severe oxidative damage to cell membranes caused by cold stress. In contrast, the M15-inoculated CB9 group exhibited significantly lower MDA levels at 11.94 ± 1.31 nmol/g, 17.77 ± 3.53 nmol/g, and 21.19 ± 4.14 nmol/g for LL, SL, and TTL, respectively (Figure 7.1A). Similarly, SOD activity in the control group was measured at 530.58 ± 79.02 U/g, 503.17 ± 49.24 U/g, and 473.48 ± 174.64 U/g for LL, SL, and TTL, respectively (Figure 7.1B). The M15-inoculated group showed significantly increased SOD activity, measured at 805.90 ± 34.12 U/g, 575.21 ± 81.14 U/g, and 596.89 ± 61.36 U/g for LL, SL, and TTL, respectively (Figure 7.1B). In the booting stage, rice varieties treated with two methods exhibited significant phenotypic differences (Figure 7.1C). CB9, as the control group, displayed weak stems, yellowing and wilting leaves, and showed slower growth. In contrast, the CB9+M15 inoculated group demonstrated markedly enhanced characteristics, including stronger stems, greener leaves, and notably improved growth. This indicates that M15 inoculation significantly improved the phenotypic performance of the CB9 variety in the booting stage, enhancing plant growth and development, especially under cold stress.

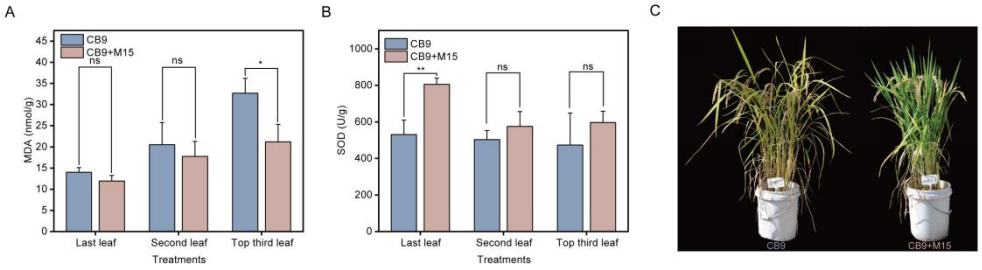

Figure 7.1 Effects of M15 inoculation on MDA content and SOD activity in the leaves of CB9 rice during the booting stage. (A) Changes in MDA (malondialdehyde) content in the last leaf (LL), second leaf (SL), and top third leaf (TTL) of CB9 control and M15-inoculated groups. (B) Changes in SOD (superoxide dismutase) activity in the last leaf (LL), second leaf (SL), and top third leaf (TTL) of CB9 control and M15-inoculated groups.（C） The picture shows the phenotypic comparison of CB9 rice without inoculation and CB9 rice inoculated with M15 at the booting stage. CB9: Non-inoculated CB9 rice (control group). CB9+M15: M15-inoculated CB9 rice (experimental group). Error bars indicate standard errors. ns indicates no significant difference ($p > 0.05$); * indicates significant difference ($p < 0.05$); ** indicates highly significant difference ($p < 0.01$).

These findings demonstrate that M15 inoculation effectively mitigates oxidative damage caused by cold stress in CB9 rice and enhances cold tolerance by increasing SOD activity. Previous studies have shown that cold stress induces excessive ROS production in plants, leading to lipid peroxidation and cellular damage (Gill and Tuteja, 2010; Hasanuzzaman et al., 2020). In this study, the significant reduction in MDA content in M15-inoculated plants indicates that M15 alleviated membrane

lipid oxidative damage caused by cold stress. SOD plays a critical role in the antioxidant system by scavenging superoxide anions (O^{2-}), maintaining redox balance, and mitigating oxidative stress (Hossain et al., 2015). The observed increase in SOD activity in the M15-inoculated group suggests that enhanced antioxidant capacity is a key mechanism for the improved cold tolerance of CB9 rice. Previous research has reported that certain endophytic bacteria enhance plant stress tolerance by modulating the host's antioxidant system (Kamran et al., 2022; Ameen et al., 2024). It is plausible that M15 exerts its effects by secreting metabolites or activating antioxidant-related gene expression in rice, thereby reducing ROS-induced oxidative damage during cold stress. This mechanism aligns with previously reported plant-microbe interaction models (Inbaraj, 2021). The growth phenotypic differences of CB9 rice under different bacterial treatments indicate that M15 inoculation has a pronounced positive effect on rice growth during the booting stage, particularly in terms of cold tolerance and adaptation to low temperatures. The CB9 variety, which is generally poor in cold tolerance under clod stress, showed improved cold tolerance after M15 inoculation, resulting in better growth performance under cold conditions. These findings are consistent with previous studies during the seedling stage, confirming that endophytic bacterium plays a crucial role in enhancing plant stress tolerance. Compared to traditional cold adaptation methods, microbial inoculation as a biotechnological approach has shown greater potential in improving plant growth and cold tolerance.

7.3.1.2 Growth parameter changes in rice under cold stress at the booting stage

Following the cold stress treatment, rice plants were cultivated until maturity, and grain yield and growth parameters were measured. These parameters included plant height, panicle length, panicle number, 1,000-grain weight, filled grain weight, and unfilled grain weight. Results showed that plant height in the M15-inoculated group was 95.00 ± 5.66 cm, significantly higher than 89.00 ± 2.83 cm in the control group (Figure 7.2A). Panicle length was also significantly increased in the M15 group, measured at 18.30 ± 2.88 cm compared to 16.98 ± 3.04 cm in the control group (Figure 7.2B). The number of panicles per plant in the M15 group was 12.00 ± 1.41, compared to 16.50 ± 0.71 in the control group (Figure 7.2C). Additionally, the 1,000-grain weight was 18.20 ± 0.57 g in the M15 group, significantly higher than 16.78 ± 2.02 g in the control group (Figure 7.2D). Filled grain weight was significantly higher in the M15 group (118.42 ± 66.16 g) compared to the control group (81.27 ± 68.84 g) (Figure 7.2E), whereas unfilled grain weight was significantly lower in the M15 group (28.79 ± 21.87 g) compared to the control group (45.61 ± 32.58 g) (Figure 7.2F).

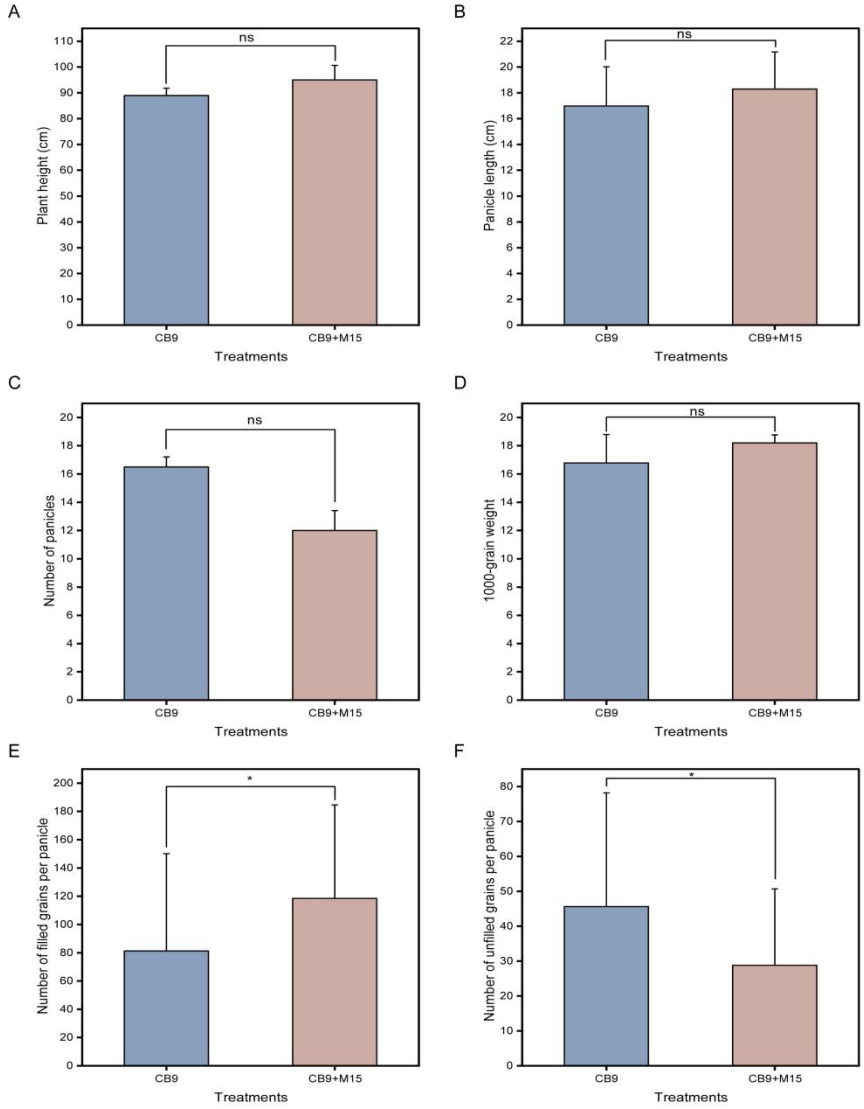

Figure 7.2 Effects of M15 inoculation on growth parameters of CB9 rice during the booting stage under cold stress. (A) Changes in plant height of CB9 control and M15-inoculated groups. (B) Changes in panicle length of CB9 control and M15-inoculated groups. (C) Changes in panicle number per plant of CB9 control and M15-inoculated groups. (D) Changes in 1,000-grain weight of CB9 control and M15-inoculated groups. (E) Changes in the number of filled grains in CB9 control and M15-inoculated groups. (F) Changes in the number of empty grains in CB9 control and M15-inoculated groups. CB9: Non-inoculated CB9 rice (control group). CB9+M15: M15-inoculated CB9 rice (experimental group). Error bars indicate standard errors. ns indicates no significant difference ($p > 0.05$); * indicates significant difference ($p < 0.05$).

The booting stage is the most cold-sensitive growth stage in rice, where cold stress inhibits vegetative growth and severely impacts panicle development and grain formation (Shimono et al., 2007). The observed increases in plant height and panicle length in the M15 group suggest that M15 significantly mitigated the negative effects of cold stress on rice growth by promoting nutrient uptake and improving plant stress tolerance. Cold stress often reduces nutrient uptake in plants; however, M15 likely enhanced nutrient absorption by solubilizing phosphate and producing plant growth-promoting substances such as IAA and siderophores, alleviating developmental inhibition caused by nutrient deficiency (Rodrí guez and Fraga, 1999; Backer et al., 2018; Fitriatin et al., 2022; Shi et al., 2024).

The increases in 1,000-grain weight and filled grain weight further confirm the positive impact of M15 on grain development. These improvements may be attributed to M15's ability to enhance the antioxidant capacity, optimize physiological metabolic states, and improve nutrient use efficiency in rice (Prasanna et al., 2012). Phosphorus, a critical element for pollen development and grain filling, was likely made more bioavailable by M15 through phosphate solubilization, providing energy support for reproductive organ development (Plaxton and Tran, 2011; Julia et al., 2016; Jeong et al., 2017; Oo et al., 2023).Additionally, M15 may have enhanced carbohydrate transport efficiency in rice, supplying more carbon resources for grain filling and contributing to higher grain plumpness (Jha et al., 2013; Doni et al., 2022).

The significant reduction in unfilled grain weight suggests that M15 mitigated cold stress-induced developmental abnormalities, likely through hormonal regulation. For instance, IAA and other plant hormones produced by M15 could stimulate plant growth and regulate grain filling processes, particularly under cold stress conditions (Teng et al., 2022; Ma et al., 2023). Moreover, M15 may have modulated the expression of cold tolerance-related genes in rice, reducing the detrimental effects of cold stress on pollen development and endosperm formation. Previous studies have shown that endophytic microbes can enhance host plant antioxidant capacity, lower ROS accumulation, and alleviate cellular damage, thereby promoting pollen viability and normal endosperm development (Huang et al., 2023). M15 likely secreted antioxidant enzymes such as SOD and CAT or carotenoid compounds to mitigate oxidative damage caused by cold stress, ensuring proper grain formation (Koza et al., 2022; Xie et al., 2022).

These results demonstrate that *M. testaceum* M15 significantly enhances the cold tolerance of CB9 rice during the booting stage. By reducing MDA content, increasing SOD activity, and improving multiple growth parameters, M15 alleviates the adverse effects of cold stress on rice physiological metabolism and growth. Additionally, M15 effectively mitigated the negative impacts of cold stress on grain development during the booting stage, contributing to improved rice yield. These findings confirm the potential of M15 to enhance crop growth and stress tolerance, providing a scientific foundation for its application as a plant biostimulant in agricultural production. The results also highlight the critical role of endophytic microbes in improving crop adaptability and productivity under environmental stresses.

7.3.2 Effects of M. testaceum M15 on the diversity of endophytic microorganisms in mature CB9 rice grains

Previous studies have shown that exogenous microbial inoculation can colonize rice plants and ultimately enter mature grains, affecting the structure and function of endophytic microbial communities (Berg and Raaijmakers, 2018; Dutta et al., 2022; Wang et al., 2023c). To confirm whether *M. testaceum* M15 can successfully colonize CB9 rice and investigate its impact on the microbial community composition in mature grains, we analyzed the diversity of endophytic microorganisms in CB9 control and M15-inoculated rice grains. This provides a scientific basis for evaluating the potential of M15 in modulating plant microbial communities.

7.3.2.1 Microbial diversity analysis

Using high-throughput sequencing of the 16S rRNA gene, we analyzed the endophytic microbial communities in CB9 control and M15-inoculated rice grains. Alpha diversity analysis revealed that M15 inoculation reduced the richness of endophytic microorganisms (considering only the presence of species) in CB9 rice grains. However, it significantly increased microbial diversity (considering both species richness and relative abundance). In the M15-inoculated group, Shannon and Observed species indices related to community richness were slightly lower than those of the control group (Figure 7.3A, 7.3B). Conversely, Shannon and Simpson indices related to community diversity were significantly higher in the M15-inoculated group compared to the control group (Figure 7.3C, 7.3D).

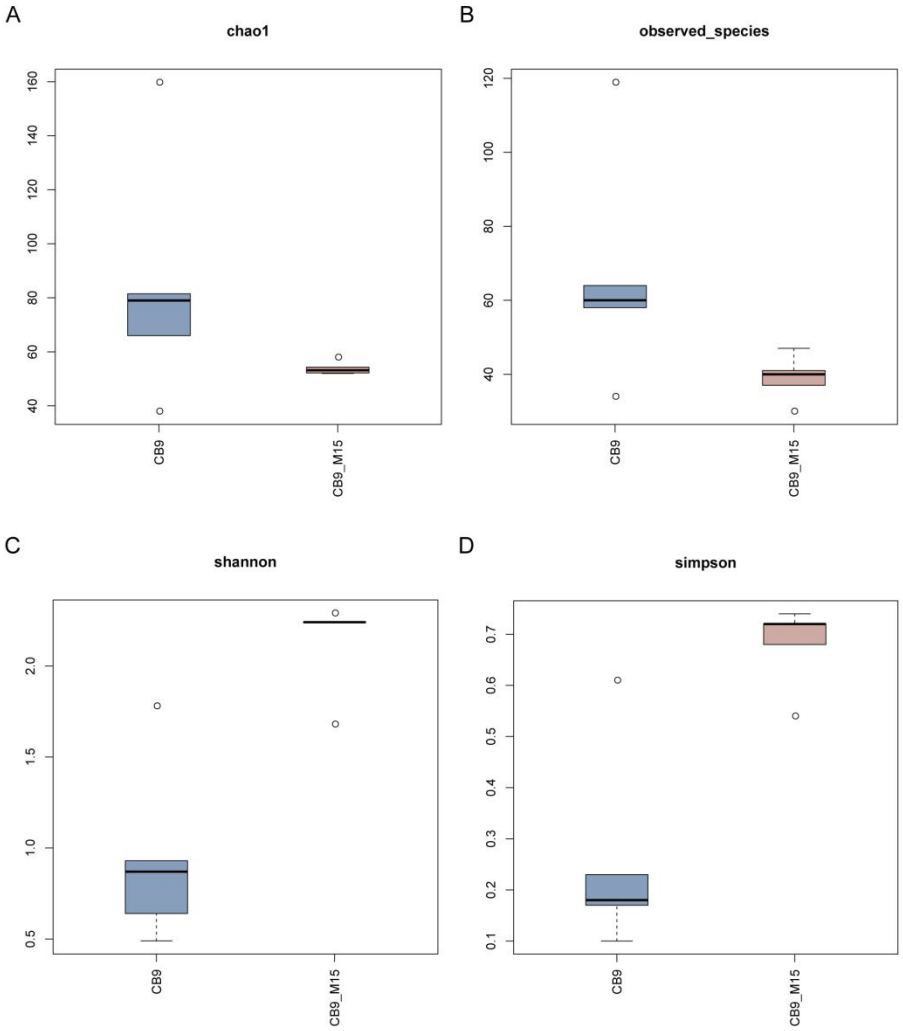

Figure 7.3 Effects of M15 inoculation on the alpha diversity of endophytic microorganisms in mature CB9 rice grains. (A) Changes in the Observed species index. (B) Changes in the Chao1 index. (C) Changes in the Shannon index. (D) Changes in the Simpson index. CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

Beta diversity analysis, including PCoA, NMDS, PCA, and PLS-DA, demonstrated that the microbial community structure in the M15-inoculated group was significantly different from that of the control group (Figures 7.4A–D). The differences in microbial composition between treatments were likely due to M15 inoculation. Specifically, PCoA based on Bray-Curtis distance indicated that M15

inoculation significantly altered the microbial community structure in CB9 rice grains (Figure 7.4A). NMDS further confirmed that microbial composition shifted significantly between treatments (Figure 7.4B). PCA revealed distinct differences between the M15-inoculated and control groups (Figure 7.4C), while PLS-DA showed clear separation between the two groups (Figure 7.4D).

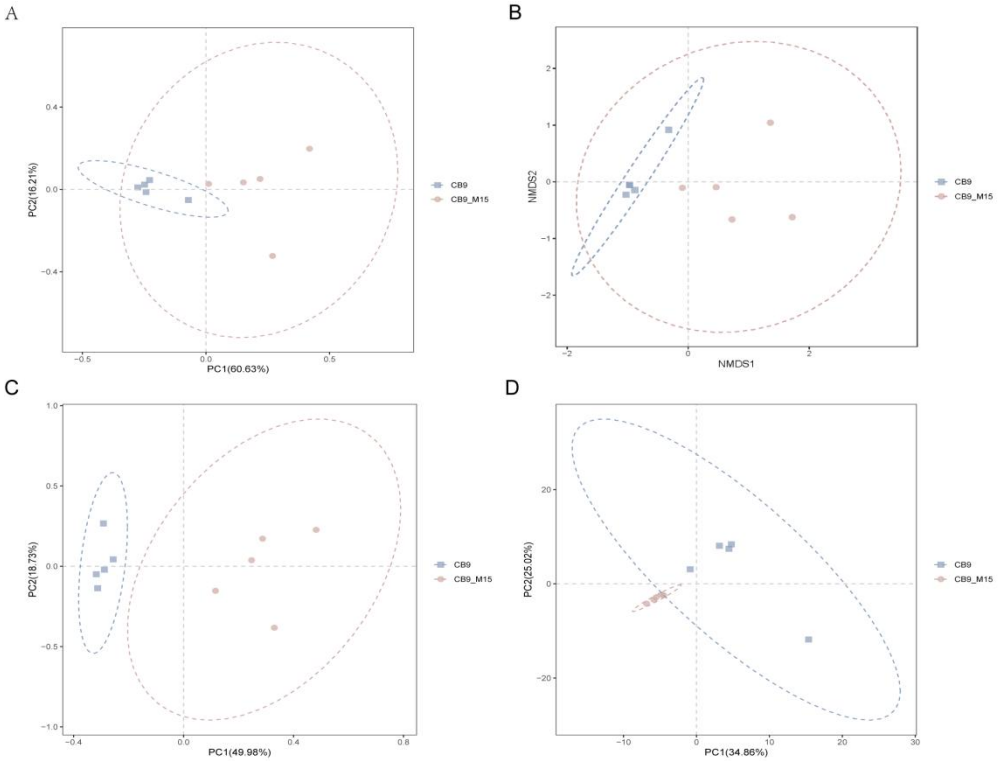

Figure 7.4 Effects of M15 inoculation on the beta diversity of endophytic microorganisms in mature CB9 rice grains. (A) PCoA (Principal Coordinate Analysis) based on Bray-Curtis distance. (B) NMDS (Non-metric Multidimensional Scaling). (C) PCA (Principal Component Analysis). (D) PLS-DA (Partial Least Squares Discriminant Analysis). CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

Taxonomic annotation analysis indicated that M15 inoculation increased the abundance of microorganisms in the family Microbacteriaceae (Figures 7.5A, 7.6A), the genus *Microbacterium* (Figures 7.5B, 7.6B), and the species *Microbacterium testaceum* (Figures 7.5C, 7.6C). The relative abundance of Microbacteriaceae increased by 67.15%, *Microbacterium* by 98.58%, and *Microbacterium testaceum* by 98.58% in the M15-inoculated group compared to the control group. These results suggest that the inoculated *M. testaceum* M15 strain significantly increased its relative abundance in the microbial community, successfully colonizing CB9 rice

and entering mature grains.

Other important genera, such as *Sphingomonas* and *Pseudomonas*, also showed increased abundance by 98.02% and 91.69%, respectively, after M15 inoculation. Conversely, the relative abundance of *Pantoea* decreased by 50.18%. These findings indicate that M15 inoculation not only successfully colonized the rice plant but also altered the structure of endophytic microbial communities in rice grains, increasing the abundance of beneficial microorganisms and reducing potential pathogens. Such optimization of the microbial community likely contributes to improved rice health and enhanced adaptability to environmental stress (Hosseiniyan Khatibi et al., 2024).

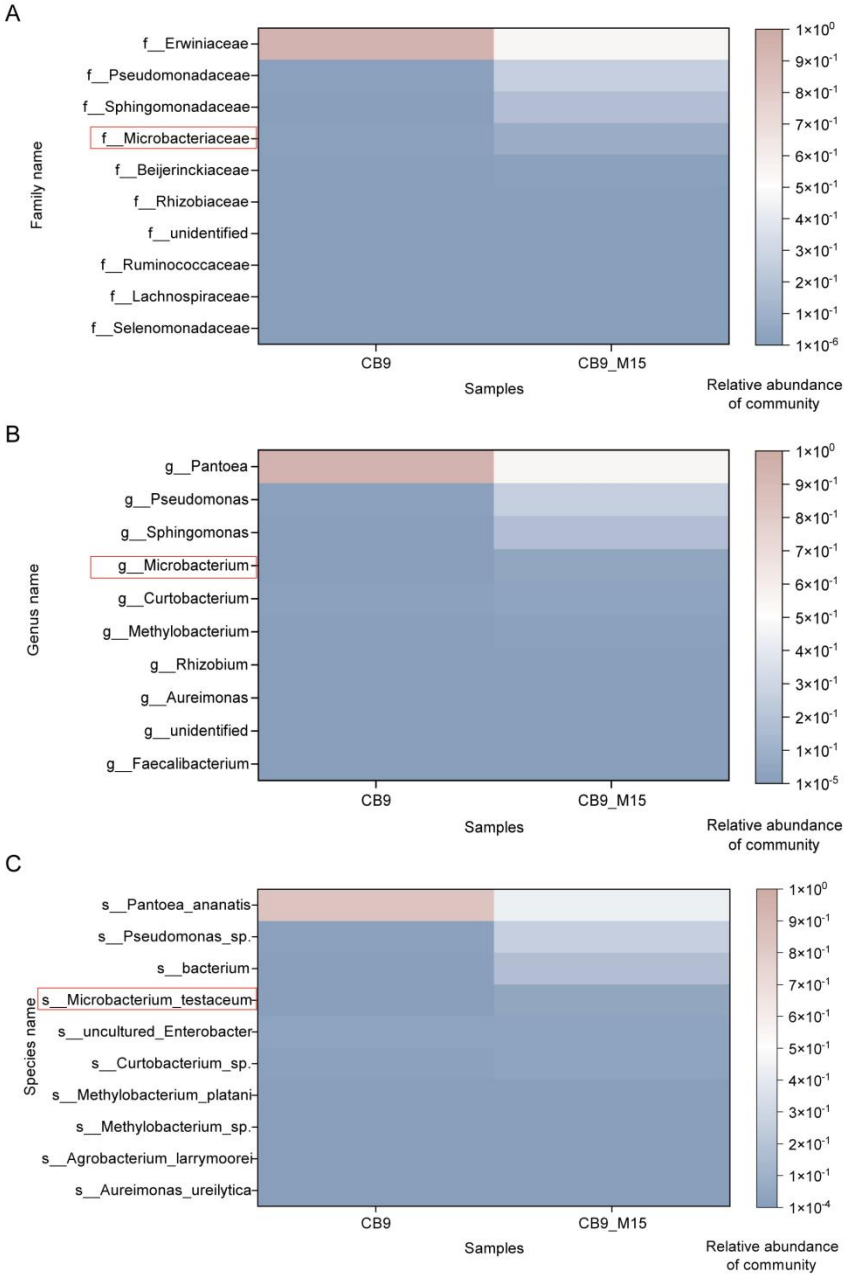

Figure 7.5 Heatmap analysis of species composition and relative abundance of endophytic microorganisms in mature CB9 rice grains after M15 inoculation. (A) Changes at the family level. (B) Changes at the genus level. (C) Changes at the species level. CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

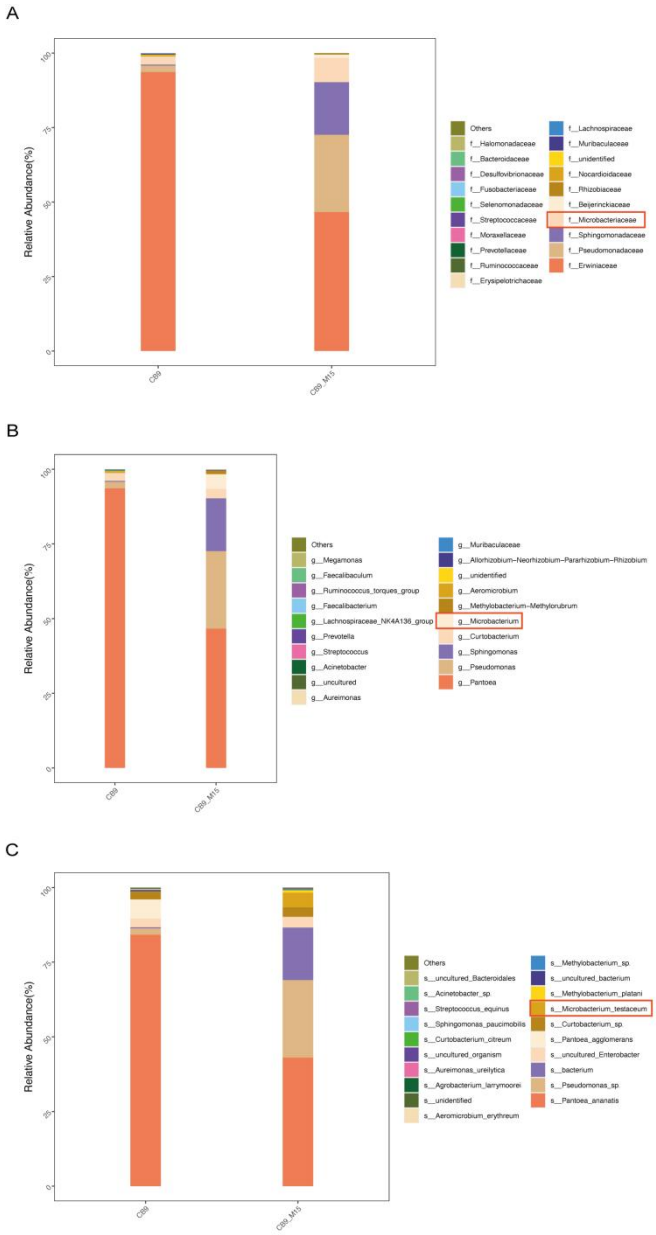

Figure 7.6 Effects of M15 inoculation on the species composition and relative abundance of endophytic microorganisms in mature CB9 rice grains. (A) Species composition and relative abundance at the family level. (B) Species composition and relative abundance at the genus level. (C) Species composition and relative abundance at the species level. CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

Using Wilcoxon rank-sum tests, we identified significant differences in microbial genera and species between the M15-inoculated and control groups. The results showed that the abundances of *Microbacterium* and *Microbacterium testaceum* were significantly higher in the M15-inoculated grains than in the control group ($p < 0.05$) (Figure 7.7). These changes underscore the critical role of M15 in the rice endophytic microbial ecosystem, demonstrating that inoculated strains can effectively colonize and alter the structure and function of host microbial communities.

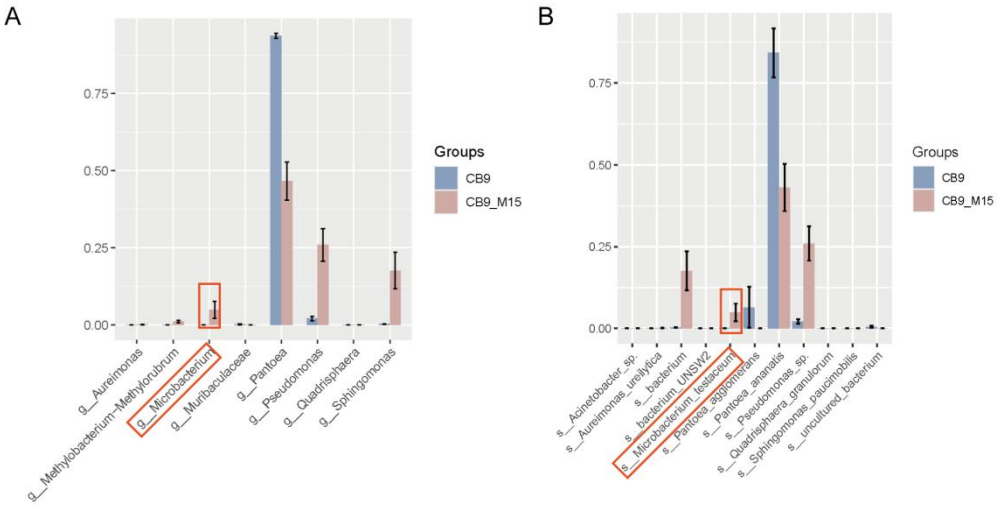

Figure 7.7 Wilcoxon analysis of significantly different microorganisms in mature CB9 rice grains after M15 inoculation. Results showing significantly enriched microorganisms at the genus and species levels. CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

Additionally, LEfSe (Linear Discriminant Analysis Effect Size) analysis further confirmed the significant enrichment of specific taxa in the M15-inoculated group. The results indicated that the LDA (Linear Discriminant Analysis) scores for *Microbacterium* and *Microbacterium testaceum* were significantly higher in the M15 group (LDA > 3, $p < 0.05$) (Figure 7.8). The enrichment of these microorganisms suggests that M15 not only successfully colonized but also played a more prominent ecological role in the inoculated group.

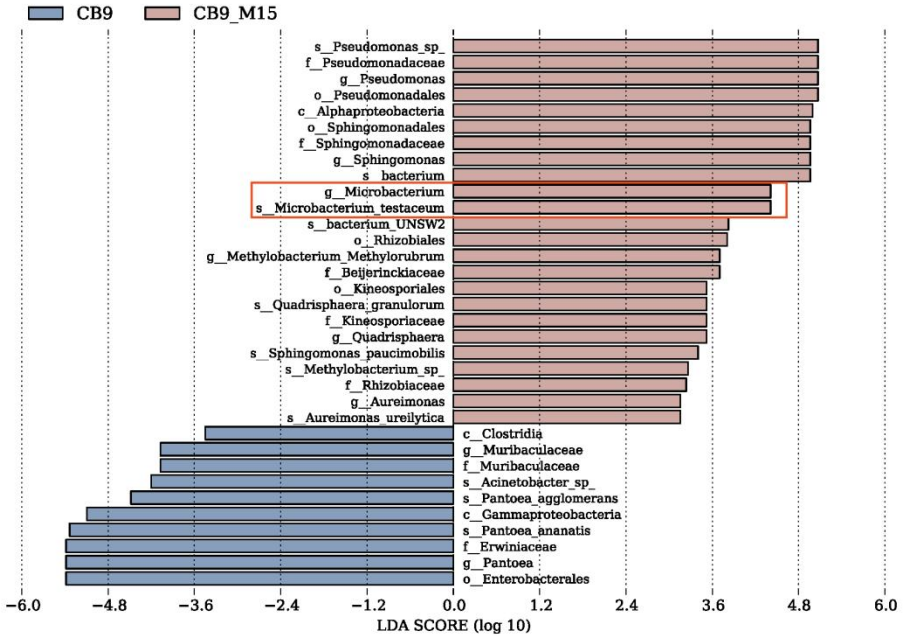

Figure 7.8 LEfSe (Linear Discriminant Analysis Effect Size) analysis of significantly different microorganisms in mature CB9 rice grains after M15 inoculation. LDA scores (LDA > 3, $p < 0.05$) of significantly enriched taxa. CB9: Non-inoculated CB9 rice (control group). CB9_M15: M15-inoculated CB9 rice (experimental group).

7.3.2.2 Functional analysis of microbial communities

The observed changes in microbial communities indicate that M15 inoculation significantly altered the endophytic microbial ecosystem in mature CB9 rice grains, optimizing the grain-associated microbiome and further enhancing plant health and productivity (Vuolo et al., 2022). These changes reflect both the direct influence of M15 on the host plant and its critical role in regulating the structure of plant endophytic microbial communities. The significant increase in *Microbacterium testaceum* is considered a key ecological effect of M15 inoculation. Previous studies have demonstrated that *Microbacterium* species not only efficiently colonize plants but also exhibit plant growth-promoting (PGP) properties and enhance stress tolerance (Tsavkelova et al., 2024).

The dominant presence of *Microbacterium testaceum* may promote rice growth and health through multiple mechanisms. Firstly, M15 can reduce ROS accumulation under cold stress by modulating the host plant's endogenous antioxidant system, thereby alleviating cellular damage. This effect has also been reported in other PGPMs, which significantly improve plant stress tolerance by activating host antioxidant enzymes such as SOD and CAT (Koza et al., 2022;

Vishnupradeep et al., 2022).

Secondly, *Microbacterium testaceum* is believed to regulate plant hormone levels. Studies have shown that *Microbacterium* species can synthesize plant hormones such as IAA and GA, which play crucial roles in controlling plant growth and development (Yadav et al., 2022). Additionally, the phosphate-solubilizing ability of *Microbacterium testaceum* may enhance phosphorus uptake and utilization in rice, promoting growth and grain filling while reducing the effects of cold stress (Fitriatin et al., 2022). Therefore, in this study, the increased abundance of *Microbacterium testaceum* may have positively influenced the growth and grain development of CB9 rice. Additionally, it may have enhanced the rice's tolerance to cold stress by modulating plant hormone signaling pathways and improving nutrient absorption and utilization.

In addition, the colonization of *Microbacterium testaceum* may suppress the proliferation of pathogenic bacteria through mechanisms such as competitive exclusion and resource occupation, thereby improving the health of rice grains (Lopes et al., 2015). As an endophytic bacterium, M15 likely occupies ecological niches within the plant rhizosphere and tissues during colonization, reducing the availability of resources and space for pathogens, thereby preventing their proliferation and infection (Zachow et al., 2008). This competitive exclusion mechanism has also been reported in other endophytic bacteria, such as rhizobacteria recruited by root exudates, which significantly lower disease incidence by efficiently colonizing the rhizosphere and excluding pathogens (Feng et al., 2024). This mechanism likely plays a critical role in M15's growth-promoting and disease-suppressive effects.

Additionally, *Microbacterium* species may secrete antimicrobial substances such as bacteriocins, volatile organic compounds (VOCs), and siderophores associated with iron competition. These substances can significantly reduce the survival and spread of pathogens by disrupting their cell walls or inhibiting their metabolic activity, directly suppressing pathogen growth (Ribeiro et al., 2021; Patel et al., 2022; Wang et al., 2023b). Studies have shown that the competitive secretion of siderophores not only inhibits pathogen growth but also enhances the host plant's tolerance to stress by improving the absorption of trace elements (Saraf et al., 2014).

Simultaneously, the inoculation of M15 may positively influence the proportion of beneficial bacteria within the endophytic microbial community of rice. This study also observed an increased proportion of other potential probiotics, such as *Pseudomonas* and *Sphingomonas*, which are known to play important roles in plant health. The synergistic effects of these probiotics may further enhance rice health and stress tolerance (Pandey et al., 2022). *Pseudomonas* species, renowned for their diverse metabolic capabilities and wide ecological adaptability, are considered key components of PGPMs. They improve plant health by secreting antibiotics, enhancing nutrient uptake efficiency, and inducing systemic resistance (ISR) in plants (Mehmood et al., 2023) Similarly, *Sphingomonas* species are recognized for their antioxidant properties, which mitigate ROS damage to plant cells and improve plant survival under stress conditions (Wang et al., 2022). Moreover, *Sphingomonas*

microbes are also regarded as plant growth-promoting bacteria, especially for their roles in regulating plant hormone balance, degrading toxic compounds, and enhancing plant tolerance to environmental stresses (Khan et al., 2017). These beneficial bacteria may interact with M15 to collectively enhance the adaptive capacity of rice to environmental stress.

These findings suggest that M15 not only establishes itself within the rice host but also optimizes the structure of the endophytic microbial community in rice grains, increasing the abundance of beneficial microbes and reducing potential pathogens. Such optimization of the microbial community may contribute to improved rice health and adaptability to environmental stresses (Hosseiniyan Khatibi et al., 2024).

7.4 Conclusion

In this study, the effects of *Microbacterium testaceum* M15 inoculation on cold tolerance and the endophytic microbial diversity of the cold-sensitive rice variety CB9 were systematically investigated during the booting stage and subsequent grain development under cold stress conditions. The results demonstrated that M15 inoculation significantly alleviated the oxidative damage caused by cold stress by enhancing the antioxidant system of rice, as evidenced by a reduction in MDA content and an increase in SOD activity in rice leaves. These physiological improvements contributed to the enhanced cold tolerance of rice during the booting stage.

Furthermore, M15 inoculation significantly improved key agronomic traits, including plant height, panicle length, 1,000-grain weight, and the number of filled grains, while reducing the number of empty grains under cold stress. These findings indicate that M15 positively influenced rice growth and yield by promoting nutrient absorption, regulating hormonal balance, and enhancing metabolic efficiency. In particular, the ability of M15 to enhance phosphorus solubilization and stimulate carbohydrate transport likely contributed to improved grain filling and overall productivity.

The analysis of endophytic microbial diversity in mature rice grains revealed that M15 inoculation successfully altered the microbial community structure. The relative abundance of beneficial microorganisms such as *Microbacterium testaceum*, *Pseudomonas*, and *Sphingomonas* was significantly increased, while the abundance of potential pathogens like *Pantoea* was reduced. This suggests that M15 not only effectively colonized the rice endosphere but also optimized the microbial ecosystem by promoting beneficial taxa and suppressing harmful ones. These changes in microbial community composition may have further enhanced the host plant's resilience to environmental stress and contributed to its overall health and productivity.

The combined results of this study highlight the multifunctional role of *Microbacterium testaceum* M15 in improving cold tolerance, promoting growth, and enhancing the microbial community of rice under adverse environmental conditions. M15 demonstrates significant potential as a biostimulant for sustainable agricultural

practices, offering a promising strategy to mitigate the impacts of abiotic stress and improve crop productivity. These findings provide a scientific basis for the future application of M15 as a microbial inoculant in agricultural production systems, particularly in regions prone to cold stress.

Chapter 8

General discussion, conclusion, and perspectives

The final chapter summarizes the main findings of the thesis, discussing the significance of these results in the context of sustainable agriculture. It suggests potential applications of microbial inoculants, particularly *M. testaceum* M15, and explores their future application directions. The chapter also addresses the limitations of the research and proposes future research directions, further investigating the role of endophytic microorganisms in enhancing crop stress tolerance.

8.1 General discussion

Rice, as an important global food crop, is widely cultivated in various climatic conditions. However, under cold stress, especially during seed germination, seedling growth, and reproductive development stages, rice often faces significant growth obstacles and yield losses. Low temperatures not only inhibit the vegetative growth of rice but also severely affect pollen development, panicle differentiation, and grain filling, thus significantly reducing the final yield of rice. Therefore, enhancing the cold tolerance of rice during cold-sensitive periods has become one of the core issues in global agricultural research.

Traditional cold tolerance improvement methods, such as breeding for cold-tolerant varieties, have made some progress to a certain extent. However, due to limited genetic diversity and long breeding cycles, there are still many challenges. Therefore, finding new and effective cold tolerance strategies, particularly enhancing cold tolerance through interactions between microorganisms and plants, has become a hot topic in plant stress biology research. In recent years, increasing research has demonstrated the great potential of microbial communities in improving plant cold tolerance. Microorganisms not only promote plant growth directly but also enhance plant tolerance to cold and other stresses by activating the plant antioxidant system, regulating nutrient absorption, and promoting metabolic pathways.

This study systematically evaluated the cold tolerance of two rice varieties JG117 and CB9 and analyzed the composition of their endophytic microbial communities at the seed and seedling stages, exploring their relationship with the cold tolerance of rice. Using high-throughput sequencing technology, we revealed the enrichment of Microbacteriaceae microorganisms in cold-tolerant rice varieties. This finding provides a new perspective on understanding rice cold tolerance and suggests that endophytic microbial communities, particularly specific types of microorganisms, may play a crucial role in enhancing rice cold tolerance.

By screening for Microbacteriaceae microorganisms from the seeds of the cold-tolerant rice variety JG117 and assessing their functional characteristics, we found that the strain *Microbacterium testaceum* M15 exhibited strong cold tolerance and plant growth-promoting traits. After detailed analysis of the M15 strain, we further revealed its multiple mechanisms of promoting plant growth under both normal and cold stress conditions. The M15 strain not only promoted the growth of cold-sensitive rice varieties (such as CB9) but also significantly enhanced their tolerance to low temperatures. Through the measurement of physiological and biochemical indicators, combined with genome and transcriptome analysis, we explored the physiological mechanisms and molecular basis through which the M15 strain enhances rice cold tolerance, including its antioxidant activity, phosphate solubilizing ability, and regulation of plant hormones. Furthermore, the potential of the M15 strain as a cold-tolerant phosphate-solubilizing microorganism was confirmed, as it optimizes rice's phosphorus absorption capacity, further improving its cold tolerance.

Finally, this study also analyzed the changes in the agronomic traits of rice inoculated with the M15 strain under cold treatment during the booting stage, as well as its effects on the microbial community of mature grains. We found that the M15 strain significantly improved the agronomic traits of rice (such as plant height, panicle length, thousand-grain weight, etc.), and effectively regulated the microbial community structure of mature grains, increasing the abundance of beneficial microorganisms and reducing the number of potential pathogenic microorganisms. These changes further enhanced the cold tolerance of rice.

Next, we will discuss the main findings of this study in detail, focusing on: the relationship between cold tolerance and endophytic microbial communities in two rice varieties; the role of the M15 strain in enhancing cold tolerance in two rice varieties; the physiological and biochemical mechanisms by which the M15 strain enhances rice cold tolerance; the genome and transcriptome characteristics of the M15 strain under different cultivation temperatures; the phosphate solubilizing mechanism of the M15 strain and the application effects of exogenous phosphorus; and the impact of the M15 strain on the agronomic traits of rice during the booting stage and the microbial community structure of mature grains. Through these discussions, we aim to further reveal the important role of the M15 strain in enhancing rice cold tolerance and provide new insights for future cold tolerance improvement in rice.

8.1.1 Microbacteriaceae microorganisms and cold tolerance in two rice varieties

In this study, we explored the cold tolerance of two rice varieties and the potential influence of their endophytic microbial communities on cold tolerance. By measuring the growth of rice under different temperature conditions, we assessed the physiological basis of two rice varieties and their cold tolerance under cold conditions. Additionally, we employed high-throughput sequencing technology to deeply analyze the endophytic bacterial communities in two rice varieties, examining the composition, abundance, and other characteristics of microorganisms and their relationship with the cold tolerance of rice.

This study first evaluated the growth performance of the rice varieties JG117 (cold-tolerant) and CB9 (cold-sensitive) under both normal and cold stress conditions. The results showed that at normal temperatures, both JG117 and CB9 exhibited similar growth patterns; however, under cold stress, JG117 exhibited significant cold tolerance advantages. After cold treatment, the survival rate of JG117 was 96%, while that of CB9 was only 8%. Moreover, JG117 showed significant advantages in root length, stem length, and fresh weight compared to CB9, further indicating that JG117 rice has stronger cold tolerance, whereas CB9 rice showed weaker tolerance to cold stress. This result is consistent with the genetic background and growth habits of the two rice varieties, especially considering that JG117 is known for its cold tolerance in Northeast China, while CB9 is more sensitive to cold stress.

To further analyze the impact of endophytic microbial communities on rice cold tolerance, we performed high-throughput sequencing analysis of the endophytic

microbial communities in the seeds and seedlings of these two rice varieties with different cold tolerances. The study found significant differences in the diversity and community structure of the endophytic bacteria between JG117 and CB9. Specifically, the endophytic microbial community in CB9 seeds had higher species richness, but under cold stress, the microbial community structure in JG117 underwent significant changes, particularly the notable enrichment of Microbacteriaceae (*Microbacterium* family). This finding suggests that the microbial community in JG117 rice may play a key role in adapting to cold stress.

Existing research has shown that rice varieties with different genotypes exhibit significant differences in their endophytic microbial communities. For example, salt-tolerant rice seeds contain a more conserved core rice microbiome and specifically enrich certain bacteria, such as *Halorhodobacter*, to adapt to saline-alkaline environments (Wang et al., 2021b). Furthermore, Denver et al. found that the host genotype significantly influences the composition of the endophytic microbial communities in rice seeds (Walitang et al., 2018). The structure of the rice seed microbiome depends on the host genotype, its physiological adaptation to salt stress, and some of the host's phylogenetic relationships. Guillermo discovered that there are differences in the types of seed-associated bacteria in drought-tolerant corn varieties compared to drought-sensitive varieties (Arellano-Wattenbarger et al., 2024). Additionally, studies have shown that the diversity of endophytic bacterial communities in hybrid rice seeds is related to their genotype, phylogenetic relationships, and rice blast disease resistance (Wang et al., 2023e). These studies indicate that changes in the structure of the rice seed microbiome are closely related to rice tolerance, particularly under biotic and abiotic stresses, where endophytic microbes can enhance rice's adaptability (Ganie et al., 2022).

Consistent with previous studies, our research also found significant differences in the microbial communities between rice varieties, and under cold stress, JG117 exhibited a high selective enrichment of Microbacteriaceae. We have discovered for the first time that this enrichment may contribute to improving rice's cold tolerance. cold stress activated specific populations within the endophytic microbial community of JG117, helping the plant cope with cold stress and enhancing its cold tolerance. Therefore, this study proposes that the structure of the endophytic microbial communities in rice seeds and seedlings may significantly influence the cold tolerance of rice, particularly the abundance differences in Microbacteriaceae, which are likely associated with the cold tolerance differences between CB9 and JG117 rice varieties.

This finding provides new insights into the symbiotic relationship between microorganisms and plants and further emphasizes the crucial role of microbial communities in plants' adaptation to cold stress. The selective enrichment of microbial communities not only helps plants cope with environmental stress but also potentially enhances plant cold tolerance by improving their adaptation to cold stress.

Although this study reveals that Microbacteriaceae microorganisms may play an important role in enhancing rice cold tolerance, this conclusion is based solely on

two rice varieties with significant differences in cold tolerance. To further validate the impact of microbial communities on rice cold tolerance, future research should expand the sample range and analyze more rice varieties with different genotypes and cold tolerance, aiming to derive more universal and widely applicable conclusions. Furthermore, future studies should explore the further effects of other microbial communities or individual microorganisms under different environmental stresses on rice cold tolerance, providing broader theoretical support and practical guidance for the application of microorganisms in agriculture.

8.1.2 Isolation, identification, and functional characteristics of Microbacteriaceae microorganisms

In previous studies, we analyzed the endophytic microbial communities of the cold-tolerant rice variety JG117 and the cold-sensitive variety CB9 under different temperature conditions using high-throughput sequencing technology. The results showed that there were significant differences in the microbial community structure between two rice varieties. Furthermore, under cold stress, the enrichment of specific microbial communities, particularly those of the Microbacteriaceae family, may play a crucial role in improving rice cold tolerance. Based on these findings, we further conducted the screening of microorganisms from the seeds of the cold-tolerant rice variety JG117 and found that the genera *Microbacterium* and *Curtobacterium* were dominant in JG117.

Further molecular identification and functional evaluation of these microorganisms revealed that in JG117 seeds, *Microbacterium testaceum* M15 exhibited significant cold tolerance and plant growth-promoting traits. Our experimental results showed that the M15 strain had a significantly higher survival rate under cold stress compared to other strains and was able to significantly improve the growth and cold tolerance of the cold-sensitive rice variety CB9. Notably, under cold conditions, the M15 strain exhibited clear plant growth-promoting characteristics, including the production of high levels of IAA, significant phosphate solubilizing activity, and the production of siderophores under both normal and cold conditions. IAA, as an important plant growth hormone, is capable of regulating plant growth and development. Phosphate solubilization and siderophore production help rice better absorb mineral nutrients, thereby further enhancing its cold tolerance.

Existing research has shown that the inoculation of exogenous microorganisms can effectively improve rice growth and tolerance under stress conditions. For example, inoculating the endophytic bacteria from drought-tolerant maize varieties into drought-sensitive maize varieties significantly increased the drought tolerance of the sensitive variety during early developmental stages (Arellano-Wattenbarger et al., 2024). Other studies have also indicated that exogenous microorganisms, once inoculated into seeds, can colonize throughout the seed's entire lifecycle and influence plant growth and development (Berg and Raaijmakers, 2018). In this study, we inoculated the *Microbacterium testaceum* M15 strain, isolated from the cold-tolerant rice variety JG117, into the cold-sensitive rice variety CB9, and the

results showed that M15 significantly promoted the growth of CB9 rice and enhanced its cold tolerance.

The growth-promoting traits exhibited by the M15 strain are consistent with those of other reported strains that promote plant growth and increase cold tolerance (Mishra et al., 2012). For example, the *Burkholderia phytofirmans* strain PsJN has been shown to stimulate grapevine growth and improve its tolerance to cold stress (Barka et al., 2006). Additionally, studies have shown that a combination of three PGPR strains can help tomatoes adapt to their environment and enhance their cold stress tolerance (Wang et al., 2016). Similarly, exogenous bacteria isolated from cold-tolerant wild plants have been shown to improve the cold tolerance of common beans (*Phaseolus vulgaris* L.) under low temperatures (Tiryaki et al., 2019). These studies suggest that microbial inoculation holds potential application value in improving crop cold tolerance, particularly in fruit trees and vegetables. Microorganisms not only promote plant growth but also effectively alleviate cold stress, thereby enhancing plant cold tolerance. For instance, inoculating cold-tolerant *Pseudomonas* can alleviate cold stress in wheat seedlings from the Northwestern Himalayas (Mishra et al., 2011b), and inoculating cold-tolerant phosphate-solubilizing *Acinetobacter rhizosphaerae* EU-KL44 can alleviate cold stress in wheat (Kour and Yadav, 2023b) . Further research has shown that the cold-tolerant phosphate-solubilizing *Serratia nematodiphila* EU-PW75 can effectively mitigate cold stress in barley (*Hordeum vulgare* L.) and promote plant growth (Kour and Yadav, 2023a). Cold-tolerant PGPRs isolated from extreme rhizosphere environments in the Qinghai-Tibet Plateau can alleviate cold stress in wheat (Zubair et al., 2019). These cold-adapted PGPB show great potential in applying microbial fertilizers under cold conditions (Li et al., 2021). However, studies on microorganisms improving rice cold tolerance are relatively few (Kakar et al., 2016), and therefore, this study contributes to filling the gap in research on microorganisms that promote rice growth and enhance rice cold tolerance.

Our research results also showed that the M15 strain not only exhibited good plant growth-promoting characteristics under normal conditions but also maintained good growth ability at 4°C. Although its ability to synthesize IAA, solubilize phosphorus, and produce siderophores at low temperatures was slightly lower than under normal conditions, it was still higher than other strains, further validating the adaptability of the M15 strain in cold environments. Furthermore, the cold tolerance of *Microbacterium testaceum* was reported for the first time in rice. Previous studies had reported the application of this strain in promoting plant growth and enhancing plant tolerance to biotic stresses, such as the *Microbacterium* Y411 associated with orchid aerial roots, which synthesizes auxin and promotes in vitro propagation (Yadav et al., 2022). Additionally, endophytic microorganisms *M. testaceum* Os-Enb-ALMB2 and *M. testaceum* OsEnb-ALM-D18 were found to exhibit antifungal activity against rice blast fungus by expressing related genes and inhibiting the activity of the rice blast fungus through the production of volatile compounds (Patel et al., 2022; Patel et al., 2023). Moreover, *M. testaceum* StLB037, isolated from potato leaves, exhibits AHL degradation activity and can effectively prevent plant pathogens (Morohoshi et al., 2011; Patel et al., 2023). Furthermore, *M.*

testaceum has also been isolated from bean leaves, where it exhibits AHL degradation activity, showing antimicrobial activity against pathogenic bacteria and inhibiting quorum sensing (Lopes et al., 2015) .

Compared to previous studies, this research further expands the application of *Microbacterium testaceum* in rice cold tolerance research, particularly the regulation of rice growth by microbial communities under cold environments. The innovation of this study lies in the fact that it is the first to reveal the potential of *Microbacterium testaceum* M15 in enhancing rice cold tolerance, providing new ideas for microbial inoculation in cold-tolerant rice varieties. However, this study focused only on cold stress and did not explore the performance of *Microbacterium testaceum* under other environmental stresses, such as salt stress and drought. Given the complexity of plant growth regulation mechanisms, future research can expand to explore the effects of M15 strains on rice growth and stress tolerance under other abiotic stresses (such as salt stress and drought). Further studies can combine multiple stress conditions to systematically evaluate the overall adaptability of the M15 strain under various environmental stresses, thereby providing broader support and rationale for the application of environmentally adaptable microorganisms.

8.1.3 Physiological and biochemical mechanisms of Microbacterium testaceum M15 in promoting rice growth and enhancing cold tolerance

In the previous work of this study, we found that *Microbacterium testaceum* M15, as a PGPM, has significant potential to promote rice growth and enhance its tolerance to low temperatures. We further investigated the role of this strain in promoting rice growth and enhancing the cold tolerance of rice seedlings, along with its physiological and biochemical mechanisms. The results indicated that the M15 strain significantly promoted rice seedling growth under normal conditions, especially in terms of root length, stem length, and fresh weight, which showed marked increases. These growth-promoting effects are closely related to the plant hormones (e.g., IAA) produced by the M15 strain, its phosphate solubilizing ability, and its capacity for siderophore production. Existing studies have shown that microorganisms can enhance plant root development and mineral nutrient absorption by generating plant growth hormones, solubilizing inorganic phosphorus in the soil, and producing siderophores, thereby promoting plant growth. For example, studies have shown that rhizosphere-promoting bacteria can produce IAA in a tryptophan-supplemented medium and possess nitrogen-fixing and inorganic phosphorus solubilizing abilities. When inoculated, they significantly increase the length of wheat shoots and roots, as well as the biomass, thereby enhancing growth and nutrient content (Majeed et al., 2015). Moreover, a phosphate-solubilizing strain, *Acinetobacter rhizosphaerae* BIHB 723, isolated from seabuckthorn rhizosphere, exhibited phosphate solubilization, auxin synthesis, ammonia production, and siderophore production, and these growth-promoting properties significantly enhanced the growth of pea, chickpea, maize, and barley under controlled conditions (Gulati et al., 2009). These results further confirm the growth-promoting

characteristics of the M15 strain, particularly its positive effect on rice growth under normal conditions.

Furthermore, the M15 strain also exhibited significant promoting effects under cold stress. We found that at 4°C, the M15 strain significantly increased rice seed germination and seedling survival rates. Specifically, the survival rate of seedlings treated with M15 was significantly higher than that of the control group, indicating that the M15 strain significantly enhanced the cold tolerance of rice under cold stress. Further physiological and biochemical analysis revealed that the M15 strain enhanced seedling chlorophyll content, total protein content, and CAT activity, thereby improving photosynthetic capacity and antioxidant ability. The M15 strain effectively alleviated oxidative damage caused by low temperatures, reducing the accumulation of MDA and mitigating oxidative damage to cell membranes, thereby protecting the cell structure and function of seedlings under cold stress.

This study further verified the potential of *Microbacterium testaceum* M15 as a PGPM in cold environments. Existing research has shown that many microorganisms help plants enhance their tolerance to stress by increasing chlorophyll content, total protein content, activating antioxidant systems, and improving membrane lipid peroxidation mechanisms. For example, the strain *Siccibacter turicensis* C2 under salt stress was able to promote barley to accumulate more proline and soluble sugars, and reduce oxidative stress by lowering hydrogen peroxide and MDA content, thereby enhancing barley's tolerance to salt stress (Sayahi et al., 2022). Additionally, *Rhodopseudomonas palustris* RP1n1 by secreting IAA and ALA, accumulating carotenoids, soluble sugars, and soluble proteins, and inducing SOD, CAT, and POD expression, also regulates plant phosphorus nutrient absorption, alleviating the harm of Tetrabromobisphenol A to soybean seedlings (Ge and Liu, 2020) . Inoculation with *Trichoderma asperellum* and *Pseudomonas fluorescens* together enhanced rice's antioxidant biochemical responses and enzyme activities, improving ROS scavenging ability and increasing rice chlorophyll content (Singh et al., 2020b) .

The innovation of this study lies in the fact that it is the first to reveal the specific mechanism by which *Microbacterium testaceum* M15 enhances rice cold tolerance, particularly in improving rice growth under cold stress by enhancing the antioxidant system and reducing oxidative damage. While this study provides strong experimental data for the application of the M15 strain in improving rice cold tolerance, some limitations remain. Although M15 significantly enhances the cold tolerance of rice seeds and seedlings under cold stress, its effect on alleviating cold stress during the later stages of rice growth still requires further investigation. Additionally, this study mainly focused on measuring and analyzing the physiological and biochemical indicators of rice under cold stress. Future studies should incorporate more physiological and biochemical indicators, along with deeper molecular mechanism studies, to comprehensively elucidate the interaction mechanisms between the M15 strain and the host plant, providing theoretical support for the application of *Microbacterium testaceum* M15 in agriculture.

8.1.4 Molecular mechanisms of Microbacterium testaceum M15 in promoting rice growth and enhancing cold tolerance

In the previous research, we explored the potential of *Microbacterium testaceum* M15 as a PGPM in promoting rice growth and enhancing its cold tolerance in seedlings. Further studies revealed the role of this strain in promoting rice growth and enhancing cold tolerance, as well as its underlying molecular mechanisms. Combining genomic and transcriptomic analysis, we revealed the gene expression characteristics of the M15 strain under both normal and cold stress conditions and identified several key functional genes associated with cold tolerance, plant growth promotion, and phosphorus metabolism. The presence of these genes provides the genetic foundation for the ability of the M15 strain to promote rice growth and enhance its cold tolerance under cold stress.

Through whole-genome sequencing analysis, we found that the genome of M15 contains multiple key genes associated with environmental adaptation, plant growth promotion, and cold tolerance. These genes include CSPs, HSPs, antioxidant-related genes (such as SOD, CAT), genes involved in the synthesis of osmoprotectants (such as betaine and proline), and genes related to EPS synthesis. The roles of cold shock proteins and heat shock proteins have been widely reported; these proteins help cells cope with stress, such as low temperatures, and protect cell structure and function. Previous studies have shown that CSPs and HSPs play important protective roles under cold stress by stabilizing the cell membrane and enhancing plant tolerance to cold (Dasila et al., 2022; Goyal et al., 2022). Similarly, the antioxidant-related genes (e.g., SOD, CAT) found in the M15 strain effectively alleviate oxidative damage caused by cold stress, maintaining normal cell function (Shen et al., 2021; Hualpa-Cutipa et al., 2022). In addition, cryoprotectants have also been shown to effectively protect cells from cold stress damage (Singh et al., 2022a; Singh et al., 2022b; Singh, 2022).

The M15 strain, due to its genetic characteristics, possesses strong cold adaptation abilities, enabling it to survive and function under cold conditions. These characteristics are consistent with reports in the literature regarding the application of cold-tolerant microorganisms in agricultural production, suggesting that the M15 strain can be used as a microbial inoculant for stress management to help crops maintain growth and enhance cold tolerance in cold environments (Yadav et al., 2019; Kushwaha et al., 2020; Puranik et al., 2022). Furthermore, the M15 strain also contains genes related to plant hormone synthesis, such as IAA synthesis genes, which promote rice growth through endogenous hormone pathways, further supporting its role as a plant growth-promoting microorganism. Genome analysis of the M15 strain also revealed the presence of genes related to siderophore synthesis, indicating its potential in enhancing nutrient availability and promoting plant health, particularly under cold conditions, which aids in increasing rice's cold tolerance (Timofeeva et al., 2022).

The M15 strain demonstrated significant phosphate-solubilizing ability by secreting organic acids (such as gluconic acid) and lowering the pH, thereby promoting the dissolution and release of phosphorus. In this study, the M15 strain

not only improved rice growth but also enhanced its ability to adapt to cold stress by increasing phosphorus utilization. Relevant studies in the literature have also reported that PSMs promote plant growth and improve stress tolerance through phosphorus solubilization (Rizvi et al., 2021). For example, Adhikari et al. (Adhikari et al., 2021) reported that *Pseudomonas* strains isolated from high-altitude soils in the Himalayas possess phosphorus solubilizing potential under low temperatures, promoting the growth of *Arabidopsis*. Subramanian et al. (Subramanian et al., 2016) found that cold-tolerant phosphate-solubilizing bacteria isolated from overwintering soils could improve tomato cold tolerance under cold stress, characterized by reduced membrane damage, activated antioxidant enzymes, and increased proline synthesis. Similarly, Yarzábal et al. (Yarzábal et al., 2018) isolated cold-tolerant *Pseudomonas* strains from Antarctic soils, which significantly improved the root and shoot length of wheat seedlings under cold conditions, further supporting the potential of M15 strain to enhance rice cold tolerance through phosphorus solubilization under cold stress. Divjot et al. found that *Serratia nematodiphila* EU-PW75, a cold-adapted phosphate-solubilizing microorganism, promoted barley plant growth under cold stress, significantly improving barley's growth and physiological parameters (Kour and Yadav, 2023a).

Through further investigation of the phosphate-solubilizing mechanism of the M15 strain, we found that the strain significantly increased phosphorus solubility and promoted phosphorus bioavailability through the production of organic acids (such as gluconic acid). This mechanism is consistent with the phosphate-solubilizing mechanisms of other microorganisms reported in the literature, indicating that the M15 strain can still help rice increase phosphorus utilization under cold stress, thus enhancing rice's cold tolerance (Trivedi and Sa, 2008). However, compared to other studies on microbial phosphorus-solubilizing mechanisms, the exploration of M15's mechanism remains somewhat limited, and there may be other pathways involved in phosphate solubilization that still need further investigation to optimize the phosphorus solubilization process and improve phosphorus use efficiency.

Transcriptomic analysis showed that the M15 strain activated the Pho and Pst systems related to phosphorus metabolism under cold stress, indicating that the M15 strain improves rice's phosphorus absorption by regulating these genes, thereby enhancing its cold tolerance. This finding is consistent with studies on phosphorus-solubilizing bacteria, such as *Bacillus altitudinis* GQYP101, which regulates phosphorus bioavailability through metabolic pathways to enhance cold tolerance (Zhao et al., 2022). The Pho and Pst systems are high-affinity inorganic phosphate transporters, and the deletion of genes encoding phosphate-binding proteins in these systems significantly reduces phosphate absorption (Luz et al., 2012; Hudek et al., 2016). Based on the results from the M15 genome and transcriptome, we found significant changes in the expression of related genes under cold conditions, suggesting that M15 enhances rice's phosphorus absorption by activating these systems, thereby increasing its cold tolerance. However, further functional validation is required to establish the specific role of these genes in enhancing cold tolerance.

Additionally, we found that the addition of exogenous phosphorus under cold conditions significantly enhanced rice's cold tolerance. This result further validates the critical role of phosphorus in plant adaptation to low temperatures and supports the mechanism by which M15 improves rice phosphorus absorption through its phosphate solubilization activity. Existing studies have shown that the application of exogenous phosphorus is an effective strategy to enhance plant tolerance to cold stress (Ang et al., 2023). The addition of exogenous phosphorus enhances rice's cold tolerance by improving leaf membrane permeability and increasing antioxidant enzyme activity (Hou, 2012; Ihtisham et al., 2023). Similarly, phosphorus application reduces MDA content in wheat, mitigating membrane damage caused by MDA accumulation, and improves wheat leaf photosynthesis, increasing dry matter transport before flowering and dry matter accumulation in nutritional organs after flowering (Nie et al., 2015; Xu et al., 2022). However, excessive phosphorus fertilizer application may increase production costs and have negative environmental impacts. Therefore, using M15 as a microbial inoculant to increase phosphorus acquisition in rice provides a more environmentally friendly method of phosphorus enrichment, reducing chemical fertilizer usage while enhancing rice's cold tolerance, which aligns with the sustainability goals of modern agriculture.

Our genomic analysis revealed that Microbacterium testaceum M15 has an unusually small genome for an Actinomycetales member, suggesting an evolutionary shift toward a specialized endophytic lifestyle (Moran, 2002; Reinhold-Hurek and Hurek, 2011). Such genome reduction likely reflects loss of functions unnecessary within the protected niche of the host plant, with M15 relying on plant-derived nutrients and signals. This compact genome may underpin its efficient colonization of rice tissues and its strong growth-promoting and cold-tolerance effects. Comparative genomics with free-living Microbacterium strains and in planta expression studies of symbiosis-related genes will be essential to pinpoint the adaptations that distinguish M15 as a true endophyte..

In conclusion, this study reveals the multiple mechanisms by which *Microbacterium testaceum* M15 enhances rice cold tolerance and promotes growth. Through genome and transcriptome analysis, the M15 strain not only harbors abundant cold adaptation genes but also optimizes phosphorus absorption and utilization in rice under cold conditions, thereby enhancing rice's cold tolerance. The M15 strain successfully alleviates the negative impacts of low temperatures on rice through the synthesis of plant growth hormones, antioxidant enzymes, phosphate-solubilizing organic acids, and osmoprotectants. However, this study mainly focused on the rice model, and future research could expand to other crops, such as wheat and maize, to explore the applicability and effects of *Microbacterium testaceum* M15 in different plants. Additionally, combining more plant models to study its performance under other stress conditions would help advance microbial technology in agriculture.

8.1.5 Effect of M15 on the cold tolerance and agronomic traits of CB9 rice during booting stage and its regulation of microbial community diversity and composition in mature grains

In previous studies, we explored the role of *Microbacterium testaceum* M15 in promoting rice growth and enhancing cold tolerance, as well as its molecular mechanisms. The subsequent research focused on the impact of this strain on rice cold tolerance during the booting stage under cold stress and its regulation of microbial communities in mature grains. The results showed that the M15 strain significantly improved the growth performance and cold tolerance of rice during the booting stage under cold conditions through multiple mechanisms.

Firstly, after inoculation with M15, the MDA content in rice leaves significantly decreased, indicating that M15 could alleviate membrane lipid peroxidation and cell damage caused by low temperatures. As a marker of cell membrane damage, the reduction in MDA reflects that the M15 strain effectively mitigates oxidative damage under cold stress. Furthermore, M15 inoculation significantly increased the activity of SOD in rice leaves, enhancing the antioxidant capacity of rice and thus alleviating the oxidative damage caused by cold stress. This finding is consistent with existing research. For example, Shi et al. (Shi et al., 2024a) showed that PGPB, such as *Agrobacterium rhizogenes* and *Bacillus subtilis*, act as helper strains to synergize with *Piriformospora indica* in enhancing rice cold tolerance under cold stress. This is achieved by increasing antioxidant enzyme (such as SOD) activity and reducing MDA content, thus improving plant cold adaptation (Shi et al., 2024a). Additionally, the generation of ROS and the increase in antioxidant enzymes can play a protective role in plants, mitigating oxidative stress and enhancing cold adaptation (Baek, 2012; Mishra et al., 2023).

In terms of rice growth performance, M15 inoculation significantly promoted important agronomic traits such as plant height, panicle length, thousand-grain weight, and grain number, while significantly reducing the number of sterile grains. These improvements indicate that the M15 strain not only enhanced rice's tolerance to cold stress but also promoted its nutrient absorption and energy metabolism. Existing studies have also shown that microbial inoculation can improve agronomic traits in rice. For instance, joint inoculation with *Trichoderma asperellum* T42 and *Pseudomonas fluorescens* OKC increased growth parameters, such as tiller number, stem length, root length, and plant biomass during the grain filling period (Singh et al., 2020b). These findings further support the role of the M15 strain in improving rice growth performance and cold tolerance.

Moreover, M15 inoculation significantly altered the structure and diversity of the microbial community in rice mature grains. In the M15-inoculated group, the abundance of *Microbacterium testaceum* increased significantly, along with other beneficial microorganisms, such as *Pseudomonas* and *Sphingomonas*, while the abundance of potential pathogens decreased significantly. These changes suggest that M15 not only successfully colonized the rice endophytic environment but also regulated the microbial community structure, further enhancing rice's tolerance to stresses. Exogenous microorganisms inoculated at the seed stage colonize the rice

plant early in its lifecycle and spread as the plant grows, ultimately being detected in the mature grains at harvest. Studies have shown that this “seed-to-seed” microbial genetic transfer plays a crucial role in the assembly of plant microbiomes (Kim and Lee, 2021; Abdelfattah et al., 2023) . Moreover, existing studies have demonstrated that plants can exert long-term influences on the growth and stress resilience of subsequent generations via their associated microbiomes; such legacy effects may likewise enable M15 to continue enhancing plant cold tolerance (Bakker et al., 2020). Research also suggests that the inoculation of exogenous microbial communities can drive microbial community succession in rice, promoting plant growth and improving its ability to withstand cold stress (Zhang et al., 2024a). Additionally, some studies have summarized the functional roles of microbial symbionts in enhancing plant tolerance to cold and freezing stresses, suggesting that inoculating with synthetic microbial communities may be a critical approach to improving plant tolerance to extreme low temperatures (Acuña-Rodríguez et al., 2020).

This study primarily focused on the effects of a single microorganism (the M15 strain) as an exogenous microbial inoculant in rice, without considering the combination of synthetic microbial communities with other microorganisms, which may have certain limitations. Existing studies show that functional microbial communities may be more effective than single microorganisms in promoting plant tolerance to stress (Shayanthan et al., 2022) . Therefore, future research can consider co-cultivating M15 with other beneficial microorganisms to construct synthetic microbial communities and perform joint inoculation on rice to further analyze the synergistic effects of the synthetic microbial communities on rice growth promotion and cold tolerance.

This study systematically explored the role of *Microbacterium testaceum* M15 in enhancing cold tolerance, plant growth, and the regulation of endophytic microbial communities under cold stress during the booting stage. The results showed that M15 not only significantly improved the cold tolerance and growth performance of rice but also successfully regulated the microbial community structure in mature grains, enhancing the plant's adaptability to cold stress. As the mechanisms of M15 are further understood, its potential applications in agricultural production will continue to grow, especially in addressing the agricultural challenges posed by global climate change. The M15 strain is expected to become a key microbial tool for coping with cold stress, increasing rice yield, and stabilizing production. This study provides important theoretical support and practical guidance for the development of cold-tolerant rice and the promotion of microbial technology in agriculture.

8.2 Conclusion

Rice (*Oryza sativa* L.) is a staple crop of paramount global importance, yet its sensitivity to low-temperature stress poses a serious threat to food security. This study set out to explore an innovative microbial strategy for enhancing rice cold tolerance, focusing on the endophytic bacterium *Microbacterium testaceum* M15

isolated from seeds of the cold-tolerant variety JG117. Through a stepwise approach, from comparative microbiome profiling of two contrasting varieties (JG117 and CB9) to functional characterization, genomic and transcriptomic analyses, and reproductive-stage field simulations, we have demonstrated that M15 not only promotes rice growth under cold stress but also fundamentally reshapes the plant's physiological, biochemical, and molecular responses.

First, high-throughput sequencing of seed and seedling endophytes revealed a clear correlation between microbial community structure and varietal cold tolerance, with Microbacteriaceae markedly enriched in JG117 under low-temperature conditions. Building on this, we isolated and identified M15 for its strong IAA production, phosphate-solubilizing activity, and siderophore biosynthesis, all of which persisted, even if at reduced levels, under 4 ℃ stress. Inoculation assays confirmed that M15 improved germination rates, seedling survival, chlorophyll content, total protein levels, and antioxidant enzyme activities (e.g., CAT, SOD), while reducing lipid peroxidation (MDA) across both vegetative and reproductive stages.

Second, integrated genomic and transcriptomic analyses unveiled key gene clusters in M15, including cold-shock and heat-shock proteins, antioxidant enzymes, osmoprotectant biosynthesis pathways and high-affinity phosphorus transport systems, providing a mechanistic basis for its dual role in bacterial survival and host cold tolerance enhancement. These findings represent the first molecular dissection of *M. testaceum* M15's cold-adaptation arsenal in rice, enriching our understanding of plant-microbe symbiosis under abiotic stress.

Third, simulated field experiments during the booting stage demonstrated that M15 inoculation significantly improved agronomic traits (plant height, panicle length, thousand-grain weight) and modulated the grain-associated microbiome by increasing beneficial taxa (e.g., *Pseudomonas*, *Sphingomonas*) while suppressing potential pathogens. This "seed-to-seed" microbial legacy underscores the promise of using M15 as a seed-coating or rhizosphere amendment in cold-prone rice cultivation.

In summary, this dissertation establishes *Microbacterium testaceum* M15 as a potent endophytic ally for rice cold tolerance enhancement. By combining community profiling, functional microbiology, and multi-omics approaches, we provide a robust theoretical framework for microbial-based cold-resilience strategies. Future work should extend to large-scale field trials, explore M15's synergies with other beneficial microbes, and refine formulation and application methods to translate these findings into sustainable agricultural practice.

8.3 Perspectives

This study provides a new perspective for understanding the role of microorganisms in enhancing rice cold tolerance, particularly focusing on how microorganisms improve rice growth and physiological status under cold stress. However, despite revealing the potential of *Microbacterium testaceum* M15 in

enhancing rice cold tolerance, several unresolved questions and areas for further exploration remain. Future research can be conducted in the following directions:

8.3.1 Microbial community diversity and ecological role

This study focused on the relationship between the endophytic microbial community in rice seeds and cold tolerance. However, the role of microbial community diversity and ecological niches in different environmental conditions and crop varieties remains an important research topic. Future research should explore the microbial communities and their functions under different environmental conditions and in various crop varieties through broader diversity studies. In particular, in-depth analysis of microbial community dynamics in rice at different growth stages and under different environmental conditions will help to reveal the comprehensive role of microorganisms in plant stress tolerance. Such research will not only enrich our understanding of microbial communities in agricultural ecosystems but also provide new ideas for crop improvement, especially in sustainable agricultural practices.

8.3.2 Microbe-plant interaction mechanisms

Although this study has preliminarily revealed the mechanisms by which *M. testaceum* M15 enhances rice cold tolerance through genome and transcriptome analysis, the specific interaction mechanisms between microorganisms and plants remain unclear. Future research could further explore how microorganisms interact with plants and examine how they increase plant tolerance through multiple pathways, such as improving antioxidant capacity and promoting nutrient absorption. This will help provide a comprehensive understanding of how microorganisms cooperate to support plant overall health, ultimately enhancing crop growth performance and environmental adaptability. Furthermore, the long-term colonization dynamics of the M15 strain within rice plants remain incompletely understood. Future research should prioritize assessing the persistence of M15 populations across different tissues and developmental stages and investigating the strength of their association with rice cold tolerance, with particular emphasis on legacy effects, to elucidate how enduring colonization may contribute to crop stress resilience.

8.3.3 Limitations of the crop model and cross-crop applicability

This study mainly focused on rice as a single crop model, investigating the potential of *M. testaceum* M15 in enhancing rice cold tolerance. Rice, as a globally important food crop, is commonly used as a representative model for plant cold tolerance studies. However, significant differences in genetic background, physiological characteristics, and responses to cold stress exist between rice and other crops, such as wheat, maize, and barley. Therefore, the broader applicability of the results remains an open question for further exploration. Future research should consider applying *M. testaceum* M15 to other crops to assess its growth-promoting, cold tolerance-enhancing effects and potential in different crops (such as wheat and maize), particularly evaluating its adaptability in regions with fluctuating climates,

diverse soils, and varying agricultural management conditions.

8.3.4 Responses to different types of environmental stresses

This study mainly focused on cold stress, but other environmental stresses and inoculation, such as salinity, drought, and heavy metal pollution, also pose significant challenges to agricultural production. Future research should expand the application of microorganisms under multiple environmental stresses, exploring how they respond to and mitigate these environmental pressures. Especially in the context of climate change deeply impacting agricultural production, microorganisms, as part of sustainable agricultural technologies, demonstrate great application potential. Developing diverse microbial solutions will become an important research direction for future agricultural production. This includes testing the performance of microorganisms under various abiotic stresses and assessing their long-term effects on crop yield.

8.3.5 Field trials and practical application

Although this study has demonstrated the effectiveness of *M. testaceum* M15 in pot experiments, field trials are crucial for assessing its practical applications. Future research should expand the scale of experiments and conduct field trials to evaluate the effects of different microbial strains under various climate, soil, and agricultural management conditions. To facilitate the field application of *M. testaceum* M15, we recommend the following strategies: seed coating with the bacterial formulation, rhizosphere soil drenching at transplanting, foliar spraying during critical growth stages, and co-application with phosphorus fertilizers to enhance both colonization and nutrient uptake. Field trials will not only help understand how microbial treatments interact with other agronomic practices (such as irrigation, fertilization, and crop rotation) to enhance rice cold tolerance, but should also explore the economic feasibility and operability of microbial applications. Specifically, how to develop low-cost, scalable microbial products that can be widely applied in agricultural production, thereby promoting sustainable agriculture.

In conclusion, this study provides a theoretical foundation for understanding the role of microorganisms in enhancing rice cold tolerance and offers new insights into the agricultural application of microorganisms. Future research should continue exploring various aspects, including microbial functions, plant-microbe and PGPR-microbiota interaction mechanisms, and environmental adaptability. With continuous advancements in technology, microorganisms will play an increasingly important role in global agricultural sustainability, particularly in addressing the challenges that climate change poses to agriculture. The results of this study provide an important theoretical basis and practical guidance for the development of cold-tolerant rice and the promotion of microbial technologies in the agricultural sector.

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Appendix

Articles

1. **Zhao J**[1], Liu X[1], Hou L, Xu G, Guan F, Zhang W, Luo H, Wu N, Yao B, Zhang C, Delaplace P, Tian J. **(2024).** The seed endophytic microbe *Microbacterium testaceum* M15 enhances the cold tolerance and growth of rice (*Oryza sativa* L.). Microbiological Research 2024;289:127908. https://doi.org/10.1016/j.micres.2024.127908

2. **Zhao J**[1], Yu X[1], Zhang C, Hou L, Wu N, Zhang W, Wang Y, Yao B, Delaplace P, Tian J. **(2023).** Harnessing microbial interactions with rice: Strategies for abiotic stress alleviation in the face of environmental challenges and climate change. Science of The Total Environment 2024;912:168847. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.168847

3. Yu X[1], **Zhao J**[1], Ding Z, Xiong F, Liu X, Tian J, Wu N. **(2023).** Cadmium-absorptive Bacillus vietnamensis 151–6 reduces the grain cadmium accumulation in rice (*Oryza sativa* L.): Potential for cadmium bioremediation. Ecotoxicology and Environmental Safety 2023;254:114760. https://doi.org/https://doi.org/10.1016/j.ecoenv.2023.114760

4. Yu X[1], **Zhao J**[1], Liu X, Sun L, Tian J, Wu N. **(2021).** Cadmium Pollution Impact on the Bacterial Community Structure of Arable Soil and the Isolation of the Cadmium Resistant Bacteria. Frontiers in Microbiology 2021;12. https://doi.org/10.3389/fmicb.2021.698834

5. **Zhao J**[1], Liu X[1], Hang D, Wu N, Tian J. **(2021).** Screening Method of Functional Microorganisms Interacting with Rice Roots. Bio-101 2021:e2003638. https://doi.org/10.21769/BioProtoc.2003638 (Chinese periodicals)

Patents

1. Xiaoqing Liu, **Jintong Zhao**, Feifei Guan, Ningfeng Wu, Jian Tian, Guoshun Xu, Yaru Bao. *Microbacterium testaceum* for Promoting Rice Growth and Enhancing Seedling Cold Tolerance and Its Application. **(2022).** ZL202210363880.3 China Patent

Scientific communications

1. **Jintong Zhao**, Jian Tian, Pierre Delaplace, Chunyi Zhang, and Ningfeng Wu，et al （2023）Bacillus megaterium 165-2 isolated from cadmium soil can promote rice growth and alleviate cadmium stress. *Dignified Researchers in Agricultural, Biological & Life Sciences Conferences*. Madrid, Spain. (Oral presentation)

2. **Jintong Zhao**, Jian Tian, Pierre Delaplace, Chunyi Zhang, and Ningfeng Wu，et al （2023） The phosphate-solubilizing bacterium *Microbacterium testaceum* MT15 can improve the growth and cold tolerance of rice. *Translational Research in Crops Research*. Ghent, Belgium. (Poster)