Improving Cold Tolerance of Asian Rice through Plant Growth Promoting Bacteria and Rice Gene Manipulation: Mechanisms of Pseudomonas Mosselli and OsMADS27

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IMPROVING COLD TOLERANCE OF ASIAN RICE THROUGH PLANT GROWTH PROMOTING BACTERIA AND RICE GENE MANIPULATION: MECHANISMS OF *PSEUDOMONAS MOSSELII* AND *OSMADS27*

by

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ABSTRACT
IMPROVING COLD TOLERANCE OF ASIAN RICE THROUGH PLANT GROWTH PROMOTING BACTERIA AND RICE GENE MANIPULATION: MECHANISMS OF *PSEUDOMONAS MOSSELII* AND *OSMADS27*

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It is estimated that by the year 2050, the human population will extend to 9.7 billion and therefore the demand for food will increase. It is important to find ways to maintain production of critical crops, like rice, to match the demand. Climate change affects crop productivity as it is the reason for extreme warm or cold weather fluctuations. Asian rice (*Oryza sativa* L.) generally is grown in tropical and subtropical regions and thus, is sensitive to cold temperatures. This study hypothesized two strategies could improve growth and cold tolerance of rice plants: (1) use of plant growth promoting bacteria (PGPB), (2) gene manipulation of rice plants. To test the hypothesis concerning PGPB, a total of 140 bacteria were isolated and characterized from the root surface and inner tissues of roots and shoots of two-week old *indica* and *japonica* rice plants. Out of the 140, 5 PGPB were selected, based upon their characteristics, for testing their *in vivo* plant growth promoting capabilities to improve rice cold tolerance. Results showed that 4 of these PGPB, *Pseudomonas mosselii*, *Paenibacillus rigui*, *Paenibacillus graminis* and *Microvirga* sp. improved growth in a rice genotype-dependent manner but only *P. mosselii*, improved both *indica* and *japonica* varietal plants' cold survival through increasing antioxidants such as reduced glutathione and proline, respectively. Additionally, *P. mosselii* showed antagonisms against phytopathogens, *Fusarium fujikuroi* and *Talaromyces aurantiacus*, and improved cold survival of *japonica* plants through either production of secondary metabolites or upregulation of defense genes. The data supported the hypothesis that *P. mosselii* contributes to cold tolerance of *O. sativa* through different mechanisms depending on the rice genotype. For the second hypothesis, *OsMADS27* was chosen to evaluate its influence on cold tolerance and interactions with *P. mosselii*. Results indicated that *OsMADS27* stimulated defense and peroxidase genes to detoxify excess amounts of reactive oxygen species (ROS) in rice plants as a response to cold stress. Furthermore, the presence of *P. mosselii* increased the cold survival of *OsMADS27* overexpressed *indica* plants. Further studies need to be performed to understand the interactions of *OsMADS27* overexpression with *P. mosselii* in improving cold tolerance of rice plants.

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Chapter 1: Background and Significance

1.1 Plant growth promoting (PGP) microorganisms

Soils are rich environments for both plants and microbial communities (Kaku et al., 2008), with the latter having a wide biological diversity (Lupwayi et al., 1998). Thousands of microbial species can exist in only one gram of soil which is approximately more than 10 billion microorganisms (Badri & Vivanco, 2009). Soil microbes influence a variety of processes including biogeochemical cycling of nutrients, plant health and soil quality (Badri & Vivanco, 2009). The term "phytobiome" is defined as plant-associated microbiomes in which "microbiome" is described as the ecological community of commensal microorganisms, symbionts, or pathogens, which literally occupy a space in animal body systems as well as in plants (Parasuraman et al., 2019). Microbes that are involved in plant health, known as plant growth promoting (PGP) microorganisms, have beneficial interactions with plant roots. PGP microbes mostly belong to a variety of bacterial phyla including *Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Firmicutes*, Proteobacteria and *Spirochaetes* (Yadav et al., 2017). Plant growth promoting bacteria (PGPB) convert nutrients into more usable compounds for plants such as nitrogen, phosphorus, zinc and potassium; they also boost some physiological features of plants including production of growth hormones, water absorption, phytostimulation (helping plants to tolerate abiotic stress) and fighting against pathogens by producing antimicrobial compounds (Moronta-Barrios et al., 2018; Gonzalez-Mendoza et al. 2023).

Different PGPB improve growth at various life stages of plants. Atmospheric nitrogen (N_2) is not available to many eukaryotic organisms. Diazotrophic microorganisms are responsible to reduce N_2 to ammonia (NH_3) through nitrogenase enzyme and make it available to eukaryotic organisms. This process is known as nitrogen fixation. These diazotrophic microorganisms contain *nif* genes which are involved in nitrogenate synthesis. PGPB such as *Herbaspirillum* sp., *Azospirillum* sp., *Burkholderia* sp., *Pantoea* sp., *Bacillus* sp. and *Klebsiella* sp. are known to contribute to nitrogen fixation (de Souza, Ambrosini, et al., 2015). Some PGPB are involved in producing plant growth hormones (phytohormones). One of the most important phytohormones is indole-3-acetic acid (IAA). The process of IAA production in PGPB is

dependent on the existence of L-tryptophan produced by plant roots in the soil environment. Tryptophan 2-monooxygenase is an enzyme produced by IAA producing PGPB, which is dependent on tryptophan levels in the environment (Patten & Glick, 1996). However, IAA synthesis in PGPB can be tryptophan independent (B. X. Zhang et al., 2021). Some examples of IAA producing PGPB are *Enterobacter* sp., *Escherichia* sp., *Grimontella* sp., *Klebsiella* sp., *Pantoea* sp., and *Rahnella* sp. However, when plants are under stress, the amount of IAA secretion by roots will increase and this will have negative effects on growth of the plants. High concentrations of IAA will inhibit seed germination and plant growth because of ethylene production by plant roots under stressful conditions. When IAA is accumulated in plant tissues, the activity of aminocyclopropane-1-carboxylic acid synthase (ACC synthase) will be stimulated and therefore ethylene production will increase and consequently plant growth abnormalities happen (Bunsangiam et al., 2021). Furthermore, there are some pathogenic bacteria such as *Rahnella aquaticus* and *Pseudomonas syringae*, which produce IAA, and this might harm plants as well. Therefore, it is important that PGPB such as *Bradyrhizobium japonicum* and *Pseudomonas putida* with the ability of catabolizing IAA to minimize the negative effects of high concentration of IAA by producing ACC deaminase and decrease the excess amount of ethylene produced by plants under stressful conditions (de Souza, Ambrosini, et al., 2015). In addition to IAA, 1-aminocyclopropane-1-carboxylate (ACC) deaminase is another phytohormone which is produced by some PGPB including *Burkholderia* sp., *Pseudomonas* sp., *Alcaligenes* sp., *Bacillus* sp., and *Ochrobactrum* sp. (de Souza, Ambrosini, et al., 2015). Plant roots produce excess amounts of ethylene, when undesirable conditions threaten plants. In these conditions, PGPB metabolize ACC into α-ketobutyric acid and ammonia (de Souza, Ambrosini, et al., 2015). In flooded, anaerobic, and acidic soils iron toxicity might happen because of the reduction of ferric (Fe^{3+}) ions and generation of ferrous (Fe^{2+}) ions. In these cases, some PGPB develop strategies for iron uptake through siderophore usage. Siderophores are molecules with high affinity to bind or chelate Fe^{3+} ions and therefore Fe will be absorbed within bacterial cells. Some PGPB with siderophore production ability include *Enterobacter* sp., *Burkholderia* sp., *Grimontella* sp., *Klebsiella* sp., *Stenotrophomonas* sp., *Rhizobium* sp., *Herbaspirillum* sp. and *Citrobacter* sp. (de Souza, Ambrosini, et al., 2015). Phosphorus (P) is mostly insoluble in soil environment and plants uptake soluble forms of phosphorus. Some phosphate solubilizing bacteria (PSB) such as *Burkholderia* sp., *Cedecea*, *Cronobacter* sp., *Enterobacter* sp., *Pantoea*,

Pseudomonas sp., *Bacillus* sp., *Rhodococcus* sp., *Arthrobacter* sp., *Serratia* sp., *Chryseobacterium* sp., *Gordonia* sp., *Phyllobacterium* sp. and *Delftia* sp. produce siderophores, organic acids and hydroxyl ions and can solubilize inorganic soil phosphates (de Souza, Ambrosini, et al., 2015). Some PGPB such as *Acidothiobacillus* sp., *Bacillus edaphicus*, *Ferrooxidans* sp., *Bacillus mucilaginosus, Pseudomonas* sp., *Burkholderia* sp., and *Paenibacillus* sp. are able to provide potassium from potassium-bearing minerals in soil environment (Gonzalez-Mendoza et al., 2023).

To support the bacteria, plant roots provide carbon metabolites and nitrogen (Berendsen et al., 2012; Edwards et al., 2015). These compounds are known as root exudates, which can be different in each plant species at each growth stage. Therefore, each plant might attract various microorganisms depending on the genotype and growth stage of the plant (Schillaci et al., 2016).

Plant roots and their associated microorganisms can be separated into three specific zones called rhizocompartments: (1) the rhizosphere, the overall microbial community in soil associated with roots; (2) the rhizoplane, the microbial community that is in direct contact with the root surface, and (3) the endosphere (endorhizosphere), the microbial community inside the root called endophytes (Figure 1.1) (Edwards et al., 2015; Gonzalez-Mendoza et al., 2023). The rhizosphere is the area around the roots where different chemical compounds, known as exudates, are excreted from plant roots and encourage microbial growth. These exudates are produced from photosynthesis, are transported to the roots, and released to the rhizosphere as organic compounds (Aulakh et al., 2001). These organic compounds supply energy for the microbial community, and in return, beneficial microbes solubilize nutrients such as phosphorus, nitrogen and zinc in the rhizosphere and improve nutrient and water uptake to plant roots (Ilyas Dar et al., 2014).

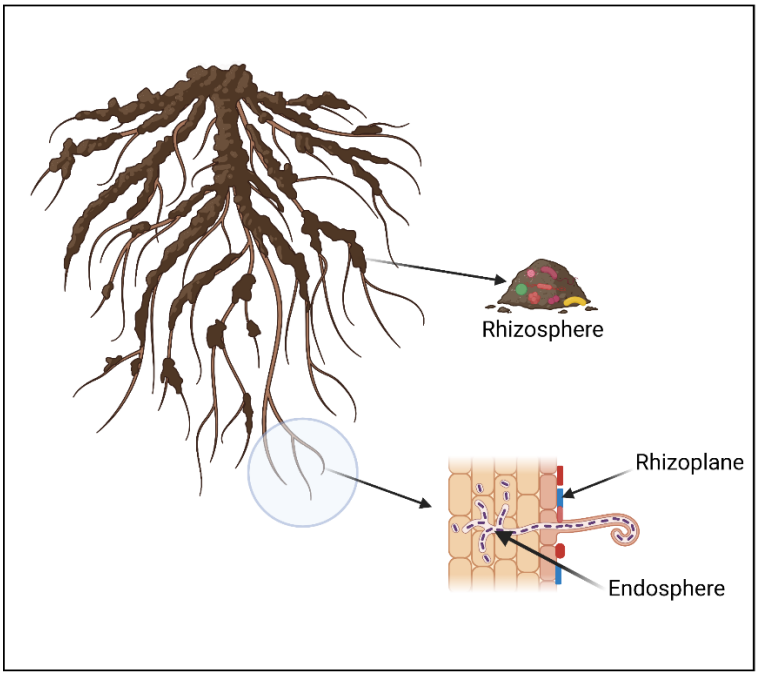

Figure 1.1 Three different plant root regions (rhizocompartments) containing microbial communities. The rhizosphere, rhizoplane and endosphere. Microbial communities in the soil attached to the root are known as rhizosphere microbes. Microbial communities attached to the root surface are known as rhizoplane microbes and microbial communities inside the inner region of roots (endosphere) are known as endophytic microbes. Figure was created with BioRender.com.

Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) analysis of plant roots have shown that the rhizoplane and the endosphere share common bacterial phyla and most of the rhizoplane bacteria are dominant in the endosphere (Vandenkoornhuyse et al., 2015). Rhizosphere and rhizoplane microbial communities have more variant abilities, while endophytic communities are more limited and specialized, with abilities such as iron acquisition and storage, degradation of aromatic compounds, detoxification of reactive oxygen species (ROS) and plant-polymer-degrading enzymes (Gonzalez-Mendoza et al., 2023). Endophytic and rhizoplane bacteria are primarily PGPB that are not only involved in plant growth, but also help the plant to better resist biotic and abiotic stress (Hassan et al., 2019). There are three types of endophytic bacteria inside plants: obligate, facultative, and passive. Obligate endophytes are transmitted via seeds and cannot proliferate outside of the plant system, while facultative endophytes are free living soil microorganisms that happen to colonize the inside of plants once the conditions are suitable for them, and passive endophytes are not actively seeking to colonize the internal tissues of plants (Gonzalez-Mendoza et al., 2023). *Burkholderia* genus is known to be an obligate and facultative endophyte (Muganu et al. 2015; Pinto-Carbó et al. 2018), and facultative endophytes

include *Azotobacter* sp., *Azospirillum* sp., *Acetobacter* sp. and *Pseudomonas* sp. (Muganu et al., 2015). Passive endophytes colonize inside plant tissues not actively and usually penetrate plant tissues through cracks and wounds along root hairs. Therefore, these types of bacteria are not as efficient as other types of endophytes in promoting plant growth (Gaiero et al., 2013). In addition to root associated bacteria, the aerial parts of plants (phyllosphere) can also be inhabited by endophytes. Some root endophytes can travel through xylem vessels in the plant and eventually inhabit the phyllosphere. These phyllosphere endophytes also benefit the plants the same ways as the root endophytes (Figure 1.2) (Parasuraman et al., 2019).

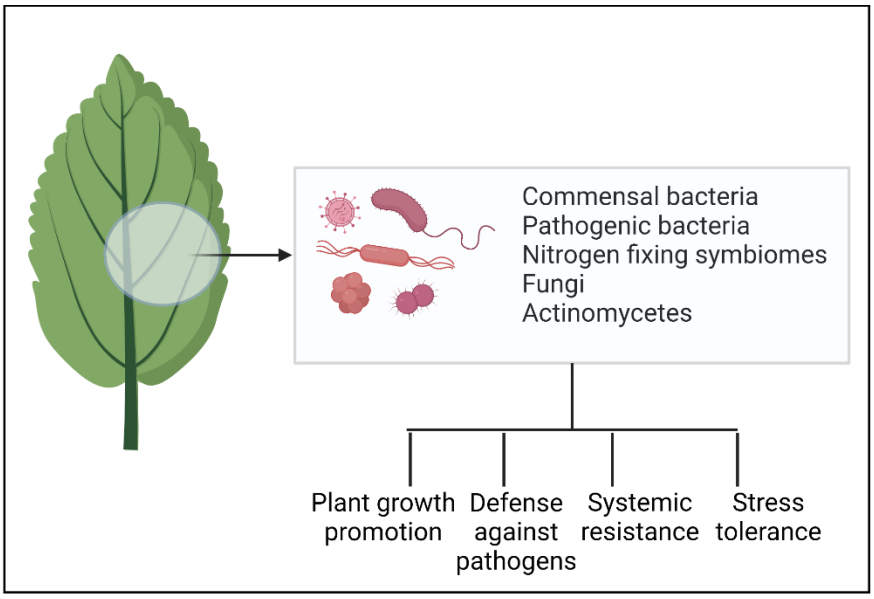

Figure 1.2 Microbial communities associated with the phyllosphere. These microbial communities are capable of benefiting plants through different roles. Figure was created with BioRender.com and adapted from Parasuraman et al. (2019).

Among plant-associated bacteria, endophytes are the most specialized because plants select these bacteria from a large microbial community that exists in the rhizosphere (Afzal et al., 2019). Depending on the plant's growth stage, endophytes are metabolically dynamic and can be found in either the roots or leaves (Gonzalez-Mendoza et al., 2023). The other factor that influences the variety of the endophytic community is the plant genotype (Reinhold-Hurek et al., 2015). Interestingly, plant-microbe interactions also depend on both plant and microbe genotypes and a PGPB that improves one type of plant growth does not necessarily promote the growth and development of other plants with different genotypes (Maghboli Balasjin et al., 2022; see also Chapter 2).

In addition to the PGPB, plants also develop their own mechanisms against biotic and abiotic stresses, such as producing phytohormones (Pessarakli, 2011). Crops are important plants that provide food and are exposed to different environmental conditions. Therefore, understanding plant defense mechanisms and the role of PGPB when the plant is exposed to stressful conditions is crucial to improve crop productivity throughout the world.

In the last 50 years, humans have been able to increase the production of food and avoid large-scale food famine. Recent development of crop varieties and management practices, known as the green revolution, have improved food production (Pessarakli, 2011). However, global climate change can cause major impacts on agriculture (Mendelsohn et al., 1994). This has led many scientists to be concerned about climate change related to the increases of both atmospheric greenhouse gas emissions and temperature of the Earth (Alpana et al., 2017). Global warming increases the occurrence of cold and warm extremes, known as the hiatus period (Johnson et al., 2018). Cold and warm extreme temperature occurrence influences crop productivity.

1.2 Chilling tolerance of plants exposed to cold stress conditions

Long winters significantly affect crop productivity because plants do not grow well in cold temperatures. Optimum temperature is the ideal one for plants which results in maximum growth and development. When plants are under cold stress conditions, their metabolic, molecular and physiological pathways will change (Yadav, 2009). Under cold stress, ROS accumulation is induced in plants which causes peroxidative reactions and therefore damages membranes, photosynthetic pigments, lipids and nucleic acids. Also, lipid composition of the membranes will be modified under cold stress and consequently the accumulation of malondialdehyde (MDA), which is the lipid peroxidation product, will occur. MDA is an indicator that shows the degree of damage to plant cells under stress conditions (Hnilickova et al. 2021; Danilo Valle Exposito et al. 2022).

To be able to survive and fight against undesirable conditions, plants have developed different response mechanisms against cold environmental conditions, that lead to improvement of their growth and development (Figure 1.3). There are two ways plants alter their metabolism when under temperature fluctuations: (1) adjusting cellular metabolism to maintain balanced metabolite levels due to alterations in catalytic properties and enzymes of plants under temperature stress conditions. In these conditions, plants

activate their regulatory mechanisms to keep normal metabolite levels and metabolite fluxes; (2) metabolism modifications in response to stress tolerance to adapt to undesirable temperatures and enhance tolerance mechanisms. There is a link between some plant metabolites such as osmolytes and stress response under temperature fluctuations. Osmolytes keep the water balance in cells as well as optimizing membrane function by distributing lipids throughout the plant cell membrane. Furthermore, gene expression pattern in plants will be modified under chilling stress (0-15˚C) (Yadav, 2009).

One of the known chilling tolerance mechanisms is producing phytohormones (plant hormones). Phytohormones regulate growth and development of plants as well as defense pathways against cold environmental conditions. Cold acclimation (CA) is one of the important defense pathways that occurs when plants are under prolonged cold stress (Pessarakli, 2011; Hassan et al., 2021). There are nonenzymatic antioxidants such as Ascorbic Acid-A (AsA), reduced glutathione (GSH), and carotenoids, which play a crucial role in plants in response to low temperatures (Pessarakli, 2016). In addition, antioxidant enzymes will be increased inside plant cells, which directly eliminate excess amounts of ROS and avoid further oxidative damage (Danilo Valle Exposito et al., 2022; Phan & Schläppi, 2021).

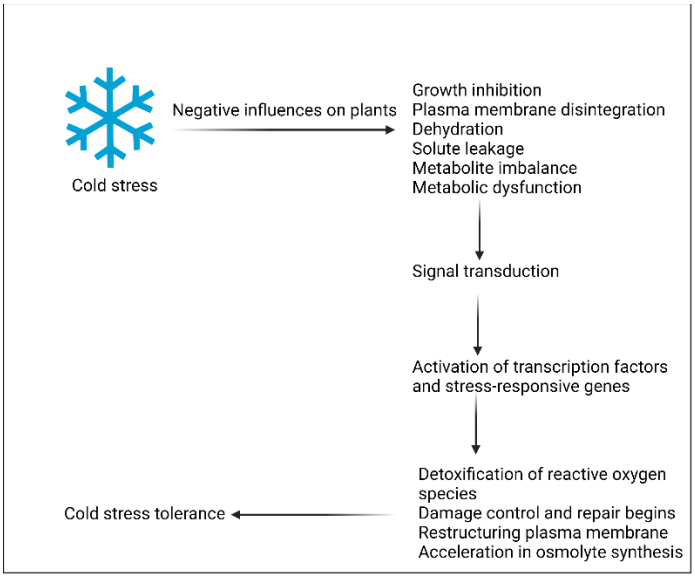

Figure 1.3 Internal plant defense responses under cold stress exposure. Cold stress has negative effects on plant growth and metabolic pathways. As a defense mechanism, plants activate genes and transcription factors to protect themselves. Figure was created with BioRender.com and adapted from Yadav, (2009).

In addition to plant defense mechanisms, different bacteria have particular importance in enhancing plants' tolerance in cold climates (Yadav et al., 2017). Studies showed that there might be an interaction between microbes and plants which causes the latter to better tolerate cold temperatures (Acuna-Rodrıguez et al., 2020; Tiryaki et al., 2019; Tibbett & Cairney, 2007).

1.3 Plant interactions with plant growth promoting bacteria (PGPB) under stress conditions

Most of the plant-microbe interactions are beneficial to both plants and microorganisms. Plant roots produce different chemical compounds known as root exudates which contain nutrition for microbial communities in the soil (Vandenkoornhuyse et al., 2015). On the other hand, microbes including bacteria and fungi impact plant growth and development through direct and indirect mechanisms (Glick, 2012). Direct mechanisms include those that provide nutrition such as soluble phosphate and zinc, nitrogen and iron, while indirect mechanisms include defense mechanisms such as antibiotic resistance and enzyme production by microorganisms to protect plants against pathogens (Glick, 2012).

Under stress conditions, growth and development of plants are inhibited or slowed down (Glick, 2012). For instance, when plants are under cold stress cell division and expansion are not efficient as well as enzyme and some protein activities (Heng Zhang et al., 2020). Furthermore, under cold stress conditions, reactive oxygen species (ROS) will accumulate in plant cells which are harmful to plants' photosynthesis, pigments, nucleic acids, lipids and membranes (Danilo Valle Exposito et al. 2022; Pang et al. 2020). To scavenge excess amounts of ROS, antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POXs), catalase (CAT) and glutathione reductase (GR) and non-enzymatic compounds such as ascorbate and glutathione will increase in plants (Tiryaki et al., 2019). In general, plants try to adapt to undesirable environmental conditions through upregulating certain stress tolerance genes. This stress-triggered cell signaling causes active growth inhibition (Heng Zhang et al., 2020). In addition to plants' defense mechanisms, different bacterial communities have particular importance in enhancing plants' tolerance in cold climates (Yadav et al., 2017). Studies showed that there might be interactions between microbes and plants which causes plants to tolerate cold temperatures (Danilo Valle Exposito et al., 2022; Acuna-Rodrıguez et al., 2020; Tiryaki et al., 2019; Tibbett & Cairney, 2007). Cold-adapted bacteria are known as psychrophilic microbes, which can grow and reproduce at low temperatures ranging from -20˚C to 20˚C. Psychrophiles mainly are members of the phyla *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* (Yadav

et al., 2019; Yadav et al., 2017). Some bacteria are known as psychrotolerant, which can grow at temperatures close to 0˚C, but their optimum growth temperature is above 20˚C and they are most likely mesophiles with a growth range from 20˚C to 45˚C (Männistö & Puhakka, 2002). These bacteria also have plant growth promoting abilities including phosphorus and zinc solubilization, nitrogen fixation, siderophore and plant hormone production that facilitate nutrient uptake and cold tolerance in plants (Verma et al., 2015). The plant microbiome in cold environments has also developed physiological adaptations (Danilo Valle Exposito et al., 2022; Yadav et al., 2017). There are various strategies such as producing compounds that activate certain protective proteins which have been developed by bacteria to tolerate cold temperatures and therefore, benefit plants under cold stress (Danilo Valle Exposito et al., 2022). Some PGPB produce 1- aminocyclopropane-1-carboxylate (ACC) deaminase to diminish the extra amounts of ethylene which is produced by plant roots under stress conditions (Tiryaki et al., 2019).

Identifying the microbiome associated with cold tolerant rice roots is crucial because plants depend upon beneficial interactions between roots and microbes for nutrient availability, growth promotion, and disease suppression (Danilo Valle Exposito et al., 2022; Lucas et al., 2014).

1.4 Priming of plant resistance by PGPB

It is estimated that the crop production can increase 12-20% through PGPB inoculation (Fiodor et al., 2023). Some PGPB are able to increase resilience of plants to undesirable environmental conditions through priming effects (Fiodor et al., 2023). Priming is described as exposing plants to stimulants to increase their tolerance (Sherin et al., 2022), which is similar to human vaccination. Priming the plants through PGPB is low-cost and low-risk and environmentally friendly (Sherin et al., 2022). In the priming effect, PGPB benefit plants through direct mechanisms such as solubilizing phosphate, nitrogen fixation and plant hormone production and therefore improve shoot and root growth and allow plants to go deep into the soil and look for more water and nutrients ahead of time, before drought, cold or salinity stresses (Fiodor et al., 2023). On the other hand, PGPB benefit plants through indirect mechanisms such as increasing ROS in plant tissues as signal molecules to synthesize more antioxidants and osmolytes such as proline, reduced glutathione and sugar before biotic and abiotic stresses harm the plants (Aranega-Bou et al., 2014).

1.5 Antifungal activity of PGPB

As mentioned earlier, indirect mechanisms which are conducted by PGPB include defense mechanisms against plant pathogens (phytopathogens) (Gutiérrez-Santa Ana et al., 2020). PGPB suppress plant pathogens through producing antifungal and bacterial volatile compounds and nutrition competition (Gutiérrez-Santa Ana et al., 2020). Fungal diseases are the most common agricultural crop diseases which spread fast (Gutiérrez-Santa Ana et al., 2020). Fungal pathogens produce spores which are able to live for a long period in soils and therefore cause different plant diseases and affect crop yield negatively. The common fungal pathogens belong to *Rhizoctonia*, *Fusarium* and *Magnaporthe* genera (Jana et al., 2022).

There are four possible mechanisms conducted by PGPB against fungal phytopathogens. (1) Some PGPB such as *Pseudomonas*, *Burkholderia* and *Bacillus* genera are capable of inhibiting fungal growth, either spore germination or hyphal growth, and suppress fungal colonization in plant tissues through emission of secondary metabolites (Jenul et al. 2018; Gutiérrez-Santa Ana et al. 2020). Secondary metabolites are compounds that are not essential for growth and are a major source of antibiotics and other bioactive compounds (Chevrette et al., 2022). *Pseudomonas tolaasii*, *Burkholderia cepacian*, *Pseudomonas fluorescens* and *Bacillus subtilis* produce different types of secondary metabolites with antifungal activity such as tolaasin, fragin, pseudomonine and fengycin, respectively (Mercado-Blanco et al. 2001; Jo et al. 2011; Jenul et al. 2018; Khadiri et al. 2023). Tolaasin can disrupt the fungal cell membrane through the formation of transmembrane pores (Coraiola et al., 2006). Fragin has metal chelating properties which inactivates fungal enzymes that use metal ions in soils with high iron concentrations (Jenul et al., 2018). Pseudomonine is a salicylic acid-based siderophore and siderophores linked to *Pseudomonas* sp. are known to restrict phytopathogen growth by sequestering iron from the environment (Mercado-Blanco et al., 2001). Fengycin is a lipodecapeptide which affects fungal cell membrane and DNA synthesis and produced by *Bacillus amyloliquefaciens* (Kulimushi et al., 2017). Some PGPB such as *Rhizobium*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, and *Aeromonas* genera are known to produce hydrogen cyanide (HCN), which is a secondary metabolite and acts as a biocontrol (Olanrewaju et al., 2017). HCN inhibits cytochrome c oxidase in phytopathogens (Blumer & Haas, 2000). (2) Some PGPB can produce enzymes such as proteases, lipases, chitinases, peroxidases and β-1,3-glucanases which target and degrade fungal cell wall compounds (Olanrewaju et al., 2017). (3) Some PGPB such as *Bacillus* genera

outcompete phytopathogens by blocking plant root binding sites or limiting nutrients in soil environment and therefore phytopathogens cannot proliferate (Olanrewaju et al., 2017). (4) Some PGPB are able to prime plants and prepare them against phytopathogens. These PGPB boost resistance mechanisms in plants through the process known as induced systemic resistance (ISR) (Olanrewaju et al., 2017).

1.6 Asian rice (*Oryza sativa* L.)

Rice is one of the most important crops worldwide and a staple food for nearly three billion people (Mccouch et al., 2016; Eizenga et al., 2019). This plant is grown mostly in soils under wet conditions, known as flooded paddy fields (Lee et al., 2015). Asian rice plants (*Oryza sativa* L.) are usually grown in tropical and subtropical areas under warm weather conditions. *O. sativa* has two subspecies including *japonica* and *indica*. Artificial selection has isolated some varieties of *O. sativa* plants that are cold tolerant (*japonica*) (Schläppi et al., 2017; Shakiba et al., 2017). *Japonica* subspecies consist of three subpopulations '*aromatic'*, '*temperate japonica*', and '*tropical japonica'* which count as cold-tolerant. *Indica* subspecies contain '*aus'* and various '*indica'* subpopulations which count as cold-sensitive subpopulations (Shi et al., 2020). *Indica* and *japonica* rice varieties have diverged in different aspects such as physiological and biochemical features, yield, quality and stress tolerance throughout years of domestication. Differences between *indica* and *japonica* varieties at the genomic level can be detected through whole genome sequencing and the application of bioinformatics. Recently, rice proteomic studies have focused on protein profiles of rice and environmental effects on gene expression (Yang et al., 2014).

It is estimated that by 2050 the world population will reach 9.1 billion and therefore agriculture production for cereal crops must increase to avoid food deficit (Lastochkina et al., 2019). In addition to human population increase, as mentioned earlier, there is a climate change that concerns both agricultural practices and scientists for crop production. Climate change increases the occurances of extreme cold weather by disrupting the polar vortex through warming temperatures and therefore bringing extreme cold temperatures to the world (Sachin Gupta et al., 2018). In order to cope with long cold days as a consequence of climate change, there are two ways to increase sustainable crop production:

(1) Use of PGPB: to have production of rice keep pace with a growing world population and climate change, the increased use of chemical fertilizers is anticipated. High amounts of fossil fuel are needed for production of nitrogen (N) fertilizers and consequently this process harms the environment,

human health, and causes environmental issues by producing nitrogen oxide which contributes to acid rains (Fields 2004; Ikeda et al. 2014). To decrease the usage of chemical fertilizers and having more sustainable rice production, biofertilizers that contain stable multispecies microbial communities should be used. There are many plant associated bacterial species in the rhizosphere, rhizoplane and endosphere of different plants that benefit crops in growth and development. Therefore, identifying the PGPB associated with different plants is crucial in this regard.

According to the review by Acuña-Rodríguez et al. (2020), there are 38 studies describing the contribution of plant associated microbes in improving growth of some plants, under cold temperatures. Very little is known about the cold-tolerant rhizoplane and endosphere bacterial isolates from rice and how they might contribute to rice cold tolerance. Until now, there have only been five studies (Danilo Valle Exposito et al., 2022; Kakar et al., 2016; Liu et al., 2014; Zhi-lei Liu et al., 2013; Redman et al., 2011) on *O. sativa* root microbial communities under cold temperatures. These studies are mostly focused on fungal species that improve rice growth in cold-stressed conditions or bacterial consortia that increase rice cold tolerance under 15˚C. Based on the studies of Redman et al. (2011), one fungus from the root endosphere known as *Curvularia protuberata* was able to enhance the germination of rice seeds in temperatures between 5 and 10˚C. Results of Liu et al. (2013, 2014) showed that there was an arbuscular mycorrhizal fungus from the root endosphere that boosted rice nutrient and water uptake at 15˚C. According to Kakar et al. (2016) there were two bacteria from rice soil rhizosphere including *Bacillus laterosporus* and *Brevibacillus amyloliquefaciens* that were able to enhance the antioxidant activity and stress-related gene expression level of plants under cold conditions (0-5˚C). Studies of Danilo Valle Exposito et al. (2022) showed that a consortium of PGPB isolated from bread wheat (*Triticum aestivum*) can positively influence growth and development of *O. sativa* through phosphate solubilization, nitrogen fixation, ammonium, hydrogen cyanide (HCN) and phytohormone (auxin) production at 15˚C. PGPB improve the cold tolerance of plants through different mechanisms such as (i) antioxidant activities by increasing ROS-scavenging enzymes (peroxidase and catalase) or proline; (ii) osmotic balance and water relations by increasing soluble sugars in plant tissues; (iii) hormonal signaling by elevating ACC deaminase, IAA, salicylic acid and β-aminobutyric acid; (iv) nutrient acquisition by increasing soluble phosphate in plant tissues (Acuña-Rodríguez et al., 2020).

There is little known about the influence of psychrophilic or psychrotolerant rice-associated rhizoplane and endosphere bacteria on the growth and gene expression of rice plants in cold conditions lower than 15˚C. Also, none of these studies compared differences between cold tolerant and sensitive rice subspecies in the matter of how specific bacteria can improve their cold tolerance.

(2) Gene manipulation of crops: rice gene manipulation started a while ago to produce stress-tolerant rice varieties in the face of climate change and a growing human population. Rice is an excellent model for genetic engineering because of its small known genome and its syntenic relationships with other cereal crops. There are differences between genetic engineering and genome editing in plants. In genetic engineering the desired gene will be isolated from the donor variety (with higher stress-tolerant and lower yield features) and will be introduced to the commercial (recepient) variety (with higher yield and lower stress-tolerant features). In genome editing the target gene (which has a negative influence on stress tolerance) will be disrupted. One specific example of genome editing technique in crops is CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) with CRISPR-associated protein Cas9 (CRISPR-Cas9) (Zafar et al., 2020).

PGPB usage as biofertilizers for rice plants and gene manipulation of rice plants have been studied separately, but not adequately in terms of cold temperature as an abiotic stressor. Furthermore, the influence of rhizosphere microorganisms on genome edited plants and mechanisms of cold tolerance have not been investigated in detail. According to Xie et al. (2022), low silicon gene 1 (*Lsi1*), one specific gene which is known for improving cold tolerance in rice plants, could increase the variety of PGPB through changing the rice root structure in the soil and therefore *Lsi1* and PGPB will improve cold tolerance of rice plants.

1.7 Transgenic rice plants and cold tolerance

To keep up rice production for a growing human population while considering climate change and environmental damage due to chemical pesticides and fertilizer usage, one way is to employ genetic engineering tools to produce genetically modified (transgenic) rice plants (Manikandan et al., 2016). Based on the different environmental conditions where rice plants are growing, different transgenic plants such as insect-resistant, herbicide-resistant, and abiotic stress-resistant can be produced. There are genome editing tools, transcriptional activator like effector nucleases (TALENs), *Agrobacterium*-mediated gene transfer

and CRISPR-Cas9 to create transgenic plants (Fu et al., 2019; Zafar et al., 2020). CRISPR-Cas9 is a suitable system for manipulating rice genes in order to produce transgenic rice plants (Jiang et al., 2019).

It is known that genetically modified crops have no negative effects on human health and the environment. There are seven transgenic rice cultivars which have been approved to be cultivated and used as a food prior to 2017 (Fu et al., 2019). However, one type of transgenic insect-resistant rice plant was commercialized as the first transgenic food crop in 2009 (Fu et al., 2019).

(Li et al., 2021)There are studies on transgenic rice plants with different overexpressed genes that improve rice plants' resistance against insects, diseases, herbicides and abiotic stresses such as salinity and drought (Bihani et al., 2011; H. Chen et al., 2018b; D. Feng et al., 2017; Fu et al., 2019; Greco et al., 2012; Gu et al., 2013; Jeon et al., 2021; Kamarudin et al., 2020; Khan et al., 2021; Kim et al., 2014; Manikandan et al., 2016; Nam et al., 2020; O'Donnell et al., 2015; Oladosu et al., 2019; Sawahel, 2002; Shanmugam et al., 2021; Shim et al., 2018; Todaka et al., 2015; Wang et al., 2005; Wang et al., 2017; Xu et al., 2020) Interestingly, studies of Nam et al. (2020) showed the possibility of transferring one gene related to drought stress tolerance in one rice variety to six other weedy rice accessions. In addition to overexpressing drought resistance genes in rice plants, studies of Shanmugam et al. (2021) showed enhancement of drought tolerance in rice plants by silencing one specific gene (*OsSYT-5*). Overexpressing one specific transcription factor (*OsMADS27*) in rice generated plants with increased salinity tolerance and nitrate uptake (Alfatih et al., 2022; H. Chen et al., 2018b). Studies of Manikandan et al. (2016) showed transgenic rice plants containing one specific protein (*cry* gene) from *Bacillus thuringiensis* became resistant to insects.

As mentioned earlier, climate change has become a major concern for scientists and agronomists, specifically prolonged winters and chilling temperatures which have negative effects on rice plants (Cruz et al. 2013; Ghosh et al. 2022). There are studies on gene modification and identification of genes in rice plants to create cold tolerant rice plants (Zhang et al. 2014, 2017; Mao et al. 2019; Guo et al. 2020; Zeng et al. 2020; Li et al. 2021). Studies by Zeng et al. (2020) showed the positive influence of three specific genes (*OsPIN5b*, *GS3*, and *OsMYB30*) in cold tolerance improvement of rice plants. Studies by Zhang et al. (2017) investigated the positive influence of *CTB4a*, which increased ATP synthase activity, on cold tolerance improvement of rice plants. Mao et al. (2019) studies showed that the *HAN1* gene in *japonica* varietal plants had a positive effect on *japonica* cold tolerance. There are other genes, transcription factors,

and proteins such as ABRE binding protein, *bZIP* transcription factors, and calmodulin-like gene such as *OsMSR2* which respond to chilling temperatures in rice plants (Z. Guo et al., 2020). According to Li et al. (2021) , there are nine studies using genome-wide association studies (GWAS) to identify quantitative trait loci (QTL) related to cold tolerance improvement in *indica* and *japonica* rice plants at different growth stages. Combining RNA sequencing (RNAseq) with GWAS analyses are efficient tools to identify QTL and associated genes related to biotic and abiotic stressors in rice plants (C. Li et al., 2021).

1.8 Hypotheses

In this PhD thesis, different aspects of rice cold tolerance have been investigated. First, the impact of PGPB, isolated from two members of *indica* and *japonica* subspecies, on different rice members of *indica* and *japonica* subspecies was evaluated. Second, cold tolerance of *Oryza sativa* was examined through PGPB inoculation. Third, antifungal activity of one specific isolated PGPB on cold tolerance of *indica* and *japonica* subspecies was investigated. Fourth, gene manipulation of *indica* and *japonica* subspecies was done to explore the influence of one specific transcription factor on cold tolerance of rice plants.

Based on above explanations, there are four hypotheses:

1. Plant growth promoting activities of PGPB are rice genotype dependent.
2. Plant growth promoting endophytes isolated from one variety of rice can contribute to the success of cold tolerance in different rice varieties through different mechanisms.
3. *Pseudomonas mosselii* controls fungal infection and improves survival of rice plants after cold stress through anti pathogen activities.
4. Overexpressing *OsMADS27*, a transcription factor, and its interactions with *Pseudomonas mosselii* increases rice cold tolerance.

1.9 Significance

Recently, the rice microbiome has become more important as a means to reduce famine and environmental damage. Regarding environmental damage, it is crucial to explore beneficial microbes to use them as biofertilizers and reduce the damage caused by chemical fertilizers. In addition, the effect of the microbiome on rice stress tolerance through different mechanisms and plant-microbe interactions are not completely known. Global climate change causes major impacts on agriculture. Long winters significantly

affect crop productivity because rice plants do not grow well in cold temperatures. While scientists have identified genetic factors involved in cold tolerance, the role of the root microbiome remains almost unexplored. On the other hand, the impacts of specific gene manipulation in rice plants, on cold tolerance of the plants, and relationships between PGPB and specific cold tolerant transcription factors needs to be investigated more in detail. This PhD thesis offers a basic understanding of microbiome-plant relationships during temperature stress, potentially unlocking improved rice growth in cold environments.

Chapter 2: Plant growth promoting activity of bacteria isolated from Asian rice (*Oryza sativa* L.) depends on rice genotype

Abstract

Asian rice is one of the most important crops because it is a staple food for almost half of the world's population. To have production of rice keep pace with a growing world population, it is anticipated that the use of fertilizers will also need to increase, which may cause environmental damage through runoff impacts. An alternative strategy to increase crop yield is the use of plant growth promoting bacteria. Thousands of microbial species can exist in association with plant roots and shoots, and some are critical to the plant's survival. We isolated 140 bacteria from two distantly related rice accessions and investigated their impact on the growth of four different rice accessions. The bacterial isolates were screened for their ability to solubilize phosphate, a known plant growth promoting characteristic, and 25 isolates were selected for further analysis. These 25 phosphate solubilizing isolates were also able to produce other potentially growth-promoting factors. Five of the most promising bacterial isolates were chosen for whole genome sequencing. Four of these bacteria, isolates related to *Pseudomonas mosselii*, *Microvirga* sp., *Paenibacillus rigui* and *Paenibacillus graminis*, improved root and shoot growth in a rice genotype-dependent manner. This indicates that while bacteria have several known plant growth promoting functions, their effects on growth parameters is rice genotype dependent and suggests a close relationship between plants and their microbial partners.

Keywords: *Oryza sativa* L., Plant growth, Plant growth promoting bacteria, Phosphate solubilizing bacteria

Importance: In this study, endophytic bacterial isolates from roots and shoots of two distantly related rice accessions were characterized phenotypically and genotypically. From the isolated bacterial species, five of the most promising plant growth promoting bacteria were selected to test their abilities to enhance growth of the four rice accessions. Interestingly, plant growth enhancement was both bacterial isolate specific and plant genotype specific. However, the positive interactions between plant and bacteria could not easily be predicted because rice growth promoting bacteria isolated from their host plants did not necessarily stimulate growth of its own host.

2.1 Introduction

Plant-microbe interactions are divided into three groups including pathogenic, symbiotic, and associative. Each of these interactions can affect plant physiology such as nutrition level, growth and development, and defense mechanisms (Ikeda et al., 2010). These interactions can be beneficial to both plants and microorganisms (Mhlongo et al., 2018). Plant-associated bacteria that benefit development of plants through direct and indirect mechanisms are known as plant growth promoting bacteria (PGPB) (A. N. Yadav, Verma, et al., 2017). Direct mechanisms of plant growth promotion include phosphorus and zinc solubilization, nitrogen fixation, plant hormone (phytohormone) production, and indirect mechanisms include antifungal activity, lytic enzyme production, siderophores and ammonia production (A. N. Yadav, Verma, et al., 2017).

There are a variety of PGPB that inhabit the area near plant roots (rhizosphere), the root surface (rhizoplane), and inside the root (endosphere) (Edwards et al., 2015). PGPB that live inside plant tissues are known as endophytes (Hardoim et al., 2008). Plants can attract specific bacteria from the soil environment to live on or inside their roots, to benefit the plants by providing and solubilizing nutrients indicating the importance of the rhizoplane and the endosphere bacterial communities (Vandenkoornhuyse et al., 2015). On the other hand, plant roots provide carbon metabolites and nitrogen to bacteria (Mhlongo et al., 2018). In addition, there are some bacteria that inhabit aerial parts of the plants known as the phyllosphere (Knief et al., 2012). Some of these phyllosphere bacteria were root endophytes that moved through xylem vessels in the plant to eventually inhabit the aerial portions (Frank et al., 2017). Phyllosphere bacteria also benefit the plants through nitrogen fixation, plant hormone production, and biotic and abiotic stress tolerance (Knief et al., 2012). The most common plant growth promoting bacteria belong to the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, *Arthrobacter*, and *Paenibacillus*. These bacteria are known to enhance plant immunity against pathogens and provide phytohormones, soluble phosphate, and/or nitrogen (Frank et al., 2017; Zhang et al., 2017; Finkel et al., 2017; Sasse et al., 2018).

Asian rice (*Oryza sativa* L.) is one of the main staple food for almost half of the world's population (Eizenga et al., 2019). It is estimated that by 2050 food demand will be heightened due to an increasing global population (Duan et al., 2012). Therefore, the rate of fertilizer use will increase, causing environmental problems (Ikeda et al., 2014). One promising solution is to create environmentally friendly bio-fertilizers containing plant growth promoting bacteria. Phosphorus is the second most important nutrient, after nitrogen, for plants but is typically insoluble in the soil (Yulianti & Rakhmawati, 2017). For plant roots to take up phosphate, insoluble inorganic phosphate needs to be converted to soluble phosphate (Chaiharn & Lumyong, 2009; Yulianti & Rakhmawati, 2017). Phosphate solubilizing bacteria (PSB) play an important role because they secrete gluconic and keto-gluconic acids and phosphatases that release soluble phosphates into the soil (Chaiharn & Lumyong, 2009; Nosrati et al., 2014; Yulianti & Rakhmawati, 2017). It has previously been shown that *Anabaena*, *Azospirillum*, *Rhodobacter* and *Streptomyces* species can promote *O. sativa* growth, but further characterization is needed to expand the known species capable of growth promotion, to understand their mechanisms of action, and their specificity for rice subspecies

(Etesami et al., 2014; Edwards et al., 2015; Yulianti & Rakhmawati, 2017). Therefore, discovering and characterizing phosphate solubilizing bacteria can improve crop productivity with less environmental impact than traditional fertilizers.

In this study, we screened for plant growth promoting bacteria by isolating bacteria from the surface and inner parts of leaves and roots of two distantly related rice varieties belonging to the two subspecies of *O. sativa*, *indica* and *japonica*. Bacterial isolates were evaluated for plant growth promoting phenotypes and the genomes of five PGPB were sequenced. Finally, we separately evaluated the effect of the sequenced isolates on growth and development of the two rice accessions from which they were isolated. In addition to these two rice accessions, we evaluated the influence of the isolated PGPB on two other rice accessions, to understand whether different rice accessions respond differently to PGPB in a matter of growth and development. To the best of our knowledge, this is the first work evaluating the influence of *O. sativa* endophytes, that were isolated from both *indica* and *japonica*, on the growth and development of members of each of the two subspecies. The endophytes were isolated from two separate varieties of two subspecies, but were tested on 4 total varieties, 2 each of the different subspecies.

2.2 Materials and methods

2.2.1 Plant growth and bacterial isolation from roots and leaves

We used accessions from the two subspecies of *O. sativa*: Krasnodarskij 3352 [Genetic Stock Oryza (GSOR) #311787] and Zhonghua 11 from the *temperate japonica* subpopulation representing the *japonica* subspecies; and Carolino 164 (GSOR #311654) and Kasalath (GSOR #301077) from the *aus* subpopulation representing the *indica* subspecies. Rice seeds were obtained from the Dale Bumpers National Rice Research Center (DBNRRC), Stuttgart, AR. Carolino 164 and Kasalath seeds were from DBNRRI field-grown plants while Krasnodarskij 3352 seeds were from plants grown in raised-bed roof top paddies at Marquette University in Milwaukee, WI, and Zhonghua 11 seeds were from plants grown at the Chinese Academy of Sciences, Beijing, China.

Following the methods of (Edwards et al., 2018), rice seeds were surface sterilized with minor modifications. Briefly, seeds were dehulled and immersed in ethanol (70% v/v in dH_2O) for 1 min, rinsed with sterile water (3X), immersed in sodium hypochlorite (70% v/v in dH_2O) for 5 min and rinsed again with sterile water (3X). After drying surface sterilized seeds on autoclaved filter papers, seeds were put into

sterile magenta boxes containing agar-solidified Murashige-Skoog (MS) medium (J. Edwards et al., 2015) for germination and incubated at 37˚C (2 d). Germinated seeds on MS media were transferred to a growth chamber with cycles of 12 h of light (28˚C) and 12 h of dark (24˚C) for 10 d. Then, seedlings were transplanted into soil pots and incubated in the same growth chamber. Soil pots contained a 50:50 mixture of two commercial soil mixes. Nutrient contents of each soil mix are listed in Table 2.1.

Table 2.1 List of two commercial soil contents used for rice growth

Contents	**Nature's care**	**Pro-Mix**
Total Nitrogen (N)	0.09%	0.30%
Ammoniacal Nitrogen	0.008%	0.01%
Other water-soluble Nitrogen	0.028%	0.04%
Water insoluble Nitrogen	0.054%	0.25%
Available Phosphate (P_2O_5)	0.08%	0.10%
Soluble Potash (K_2O)	0.09%	0.10%
Calcium	0.02%	-
	Contains 0.054% slowly available Nitrogen (N) from poultry litter alfalfa meal and kelp meal	-

Isolation of bacteria from the roots (rhizoplane and endosphere) and leaves (phyllosphere) during vegetative growth (when plants were two weeks old) was done as described previously (Edwards et al., 2018). Isolation of bacteria from roots and shoots was done with Krasnodarskij 3352 accession from the *japonica* subspecies and Carolino 164 accession from the *indica* subspecies. Roots and leaves of both rice subspecies were rinsed with a sterile phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH = 7.4 with HCl, total volume = 1 L) to remove the attached soil from roots and other contaminants from leaves. Rinsed roots and leaves were sonicated 3X in sterile PBS and surface sterilized following the methods of (Mano et al., 2007). Briefly, the roots and leaves were rinsed with sterile water, then soaked in ethanol (75% v/v in dH_2O) for 1 min and again rinsed with sterile water. Next, they were soaked in sodium hypochlorite (1% v/v in dH_2O) for 1 min and finally washed with sterile water. Sterile roots and leaves were crushed in PBS with a sterile mortar and pestle. The PBS that contained crushed roots and leaves was transferred into 1.5 mL sterile tubes and centrifuged (12,074 x g, 10 min). 100 µL of the supernatant was plated on nutrient agar (NA) media (Difco) and incubated at 30˚C. Cultures were

purified by repeatedly streaking bacteria onto NA and incubating them at 30˚C until only one colony type remained.

2.2.2 Colony and cell morphology of isolated bacteria from roots and leaves

Colony and cell morphology of isolated bacteria were determined through observing purified cultures and gram staining, respectively. Colony morphology characteristics including form, elevation, size, color, and margin were investigated (https://laboratoryinfo.com/colony-morphology-of-bacteria/; last accessed during the Spring of 2019).

Pure cultures (OD_{600} = 0.6) used for agar assay methods and spot inoculations were grown in nutrient broth (NB) medium at 30˚C for 24 h.

2.2.3 Mineral phosphate solubilization activity test

Pure cultures were grown in nutrient broth (NB) media at 30˚C for 24 h. An agar assay method was used to screen the phosphate solubilizing ability of bacterial isolates. Following the methods of (Islam et al., 2007), the National Botanical Research Institute's phosphate (NBRIP) growth medium containing glucose (10 g L^{-1}), $Ca_3(PO_4)_2$ (5 g L^{-1}), $MgCl_2$.6H2O (5 g L^{-1}), $MgSO_4.7H_2O$ (0.25 g L^{-1}), KCl (0.2 g L^{-1}) and $(NH_4)_2SO_4$ (0.1 g L^{-1}) was used for this approach. Two µL of each isolated bacterium (OD_{600} = 0.6) was spot inoculated onto NBRIP (National Botanical Research Institute's Phosphate) medium in three replicates. Plates were incubated at room temperature for 14 d. After two weeks plates were examined for phosphate solubilization activity as shown by a clear halo around the colony. The phosphate solubilization index (Islam et al., 2007) was calculated from three independent experiments as follows:

$$\text{Phosphate solubilizing index} = \frac{\text{colony diameter (cm)} + \text{halo diameter (cm)}}{\text{colony diameter (cm)}}$$

2.2.4 Screening phosphate solubilizing bacteria (PSB) for other plant growth promoting features

Both direct and indirect mechanisms of plant growth promoting screening tests were done on PSB using the following culture-based assays (also listed in Table 2.2).

Table 2.2 List of culture-based plant growth promoting assays

Direct mechanisms of PGPB assays	**Reference(s)**
Zinc solubilization	(Majeed et al., 2015)
Nitrogen fixation	(Kneen & LaRue, 1983; Jalandoni-Buan et al., 2010)
Indoleacetic acid (IAA) production (phytohormone)	(Dinesh et al., 2015)
Gibberellic acid production (phytohormone)	(Sharma et al., 2018; Gusmiaty et al., 2019)
1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity	(Majeed et al., 2015)
Indirect mechanisms of PGPB assays	**Reference(s)**
Antifungal activity	(Mehnaz et al., 2010; Dinesh et al., 2015)
Lipase production	(Mehnaz et al., 2010)
Casein and gelatin hydrolyzing (proteases)	(Rani et al., 2012; Alnahdi, 2012)
Cellulase production	(Kavamura et al., 2013)
Siderophore production	(Husen, 2003; Pahari et al., 2017)
Ammonia (NH_3) production	(Bankevich et al., 2012)

2.2.5 Zinc solubilization

Tris minimal medium (Tris-HCl 6.06 g L^{-1}, NaCl 4.68 g L^{-1}, KCl 1.49 g L^{-1}, NH_4Cl 1.07 g L^{-1}, $Na2SO_4$ 0.43 g L^{-1}, $MgCl_2.2H_2O$ 0.2 g L^{-1}, $CaCl_2.2H_2O$ 30 mg L^{-1}, agar 15 g L^{-1}) with 0.1% (w/v in distilled water) zinc sulfate ($ZnSO_4$) was prepared and 2 µL of each isolated strain (OD_{600} = 0.6) in PBS was spot inoculated onto the mentioned medium in three replicates. Plates were incubated at 30˚C for 14 d. After two weeks plates were examined for zinc solubilization activity. Similar to phosphate solubilization, a halo zone around each bacterium was confirmation for zinc solubilization (Shikha Gupta & Pandey, 2019). This experiment was repeated twice, independently.

2.2.6 Nitrogen fixation

For confirmation of nitrogen fixation, through culture-based assays, 2 µL of PSB (OD_{600} = 0.6) were spot inoculated onto nitrogen-rich yeast extract-mannitol agar containing Congo Red dye (CR-YMA) and incubated at 30˚C for 5 d. Weak-absorption of Congo Red dye by colonies was confirmation for nitrogen fixation. Bacterial colonies with colors ranging from white to pale white-pink were able to fix nitrogen (Kneen & LaRue, 1983) due to cleavage of the azo bond (-N=N-) in Congo Red (Jalandoni-Buan et al., 2010). This experiment was repeated three times, independently. *Rhizobium* sp. and *Staphylococcus aureus* were used as positive and negative controls, respectively.

2.2.7 Indoleacetic acid (IAA) production

A colorimetric assay was done using the ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) to detect IAA production. Indole compounds were quantified in precursor L-tryptophan medium. Nutrient Broth-M26 (NaCl 5 g L^{-1}, peptone 10 g L^{-1}, and beef extract 10 g L^{-1}) was used to grow bacteria for 24 h on a shaker (150 rpm) at 28˚C. 100 µL of the cultures were inoculated into 10 mL of liquid minimal salt (MS) medium (KH_2PO_4 1.36 g/l, Na_2HPO_4 2.13 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, and trace elements (containing citric acid 5 g/100 mL, $ZnSO_4.7H_2O$ 5 g/100 mL, $FeSO_4.7H_2O$ 4.75 g/100 mL, $Fe(NH_4)2(SO_4)2.6H_2O$ 1 g/100 mL, $CuSO_4.5H_2O$ 250 mg/100 mL, $MnSO_4.H_2O$ 50 mg/ 100 mL, H_3BO_3 50 mg/ 100 mL and $Na_2MoO_4.2H_2O$ 50 mg/ 100 mL in 100 mL distilled water) 1 mL) supplemented with 5 mM L-tryptophan and incubated at 28˚C for 48 h on a shaker (150 rpm). L-tryptophan medium contained glucose (10 g L^{-1}), L-tryptophan (1 g L^{-1}), and yeast extract (0.1 g L^{-1}) in 100 mL water and was filter sterilized through a 0.2 µm membrane (Whatman Syringe Filters). After 48 h of growth, 1.5 mL of bacterial solution were centrifuged at 8,870 x g for 5 min in a microfuge. One mL of the supernatant was mixed with 2 mL $FeCl_3$-$HClO_4$ reagent and after 25 min, the mixture's optical density (OD) was measured using a UV-spectrophotometer at 530 nm. A standard curve (with concentrations of 0-300 µg/mL) (Appendix A.) was created for calculating the microgram of IAA per mL of the mixture (Majeed et al., 2015). This experiment was repeated twice, independently.

2.2.8 Gibberellic acid (GA) production

GAs are other important phytohormones regulating plant growth, seed germination, and stem elongation (S. Sharma et al., 2018). Following the methods of (S. Sharma et al., 2018) and (Gusmiaty et al., 2019), the GA production detection assay was done with minor modifications. Briefly, PSB were freshly grown in NB (Difco) medium for 24 h at 30˚C, then incubated at room temperature for one week. After growth, 1.5 mL of bacterial suspensions were centrifuged (3,942 x g) for 10 min in a microfuge. One mL of supernatant was transferred into 15 mL tubes and 2 mL of zinc acetate solution (zinc acetate 21.9 g L^{-1}, glacial acetic acid 1 mL, distilled water up to 100 mL) was added to the supernatant. Two mL of potassium ferrocyanide solution (10.6% w/v in distilled water) was added to the 15 mL tubes containing supernatant and zinc acetate solution. Tubes were centrifuged (7,168 x g, 10 min) and 1 mL of supernatant was transferred to another 15 mL tube and 5 mL HCl solution (30% v/v in distilled water) was added. Tubes

were incubated 75 min at 28-30˚C after which OD_{254} measurements were taken. A standard curve (with concentrations of 0-1000 µg mL^{-1} of GA) (Appendix B.) was created for calculating the microgram of gibberellic acid per milliliter of the mixture (Sharma et al., 2018; Gusmiaty et al., 2019). This experiment was repeated twice, independently.

2.2.9 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity

A culture-based assay was done for ACC deaminase activity as described previously (Shikha Gupta & Pandey, 2019). Bacterial isolates were spot inoculated in three replicates and grown on Dworkin & Foster (DF) minimal salt medium (DF salts per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH 7.2) supplemented with 3 mM ACC as the sole nitrogen source. Isolates that grew on the plates were able to produce ACC deaminase (Shikha Gupta & Pandey, 2019). This experiment was repeated three times, independently.

2.2.10 Antifungal activity

A culture-based assay against the fungal pathogen *Magnaporthe grisea* was done as described previously (Dinesh et al., 2015), with minor modifications. Briefly, each isolate was cultured in two parallel lines (2-2.5 cm apart from each other) on potato dextrose agar (PDA) medium. *Magnaporthe grisea* causing rice blast disease was isolated from leaves of rice plants grown in rooftop paddies outside of the Schläppi lab and was spot cultured in the center of PDA plates (between two parallel bacterial lines, 0.5-1 cm away from either sides). Control plates only had the fungus without bacteria. Plates were incubated at 28˚C for five days. The radial growth of the fungus was examined to observe growth inhibition by each bacterium. Percentage of growth inhibition from three independent experiments was calculated and compared with the radial growth of fungus in control plates using the formula shown below with the following parameters: I = inhibition percentage; C = radial growth in control plate; T = radial growth in plates with bacterial isolates (Dinesh et al., 2015):

$$\mathrm{I} = \left[\frac{(C - T)}{C}\right] \times 100$$

2.2.11 Lipase production

An agar assay method was used to detect bacteria with lipase production ability. Luria-Bertani (LB) medium (tryptone 10 g, NaCl 10 g, yeast extract 5 g, agar 15 g) was prepared in 490 mL distilled water and supplemented with 1% tween-20. Two µL of each isolated strain was spot inoculated on the mentioned medium in duplicate. One set of plates was incubated at 28˚C for 3-4 d. Lipase production was detected by observing precipitation around colonies (Mehnaz et al., 2010). This experiment was repeated three times, independently.

2.2.12 Casein and gelatin hydrolyzing

Protein hydrolysis was tested using both casein and gelatin media. Casein agar medium contained NB (Difco) with 1.5% (w/v) agar. 50 mL of evaporated milk with 50 mL of sterile water were added to the sterile NB with agar (final volume = 1000 mL) and 2 µL of each isolated strain (OD_{600} = 0.6) was spot inoculated onto the medium in duplicate. Plates were incubated at 28˚C for 24 h. Casein hydrolysis was examined by observing a halo around colonies (M. U. Rani et al., 2012). Gelatin medium contained 3 g L^{-1} beef extract, 5 g L^{-1} peptone and 60 g L^{-1} gelatin in 1 L of distilled water. Bacterial solutions were stabbed into test tubes containing gelatin with inoculating needles. Tubes were incubated at 28˚C for two weeks. Bacteria with gelatinase activity were able to liquify the medium (Alnahdi, 2012). This experiment was repeated three times, independently.

2.2.13 Siderophore production

An agar assay method was used to detect bacteria producing siderophores that might help plants with iron uptake from the soil (Alnahdi, 2012), as described previously (Pahari et al., 2017). Specifically, chrome azurol S (CAS) agar was prepared by mixing four sterile solutions. Solution 1, the Fe-CAS indicator solution, contained 10 mL of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl), 50 mL of an aqueous solution of CAS (1.21 mg mL^{-1}), and 40 mL of an aqueous solution of hexadecyl-trimethylammonium bromide (HDTMA) (1.82 mg mL^{-1}). Solution 2, the buffer solution, contained 30.24 g piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) dissolved in 750 mL of salt solution (100 mL of MM9 salt solution dissolved in 750 mL of distilled water). 50 mL water was added to bring the final volume to 800 mL and 1.5% (w/v) agar was added. To dissolve PIPES in the salt solution, 50% KOH was added until the pH of the solution was 6.8. Solution 3 contained 2 g glucose, 2 g mannitol, and trace elements (same ingredients mentioned in

IAA production method section) which were dissolved in 70 mL distilled water. Solution 4 contained 10% (w/v) casamino acids, which was filter sterilized (0.2 µm membrane, Whatman Syringe Filters). All solutions, except solution 4, were autoclaved. After cooling to 50˚C, solutions 3 and 4 were mixed, then the buffer solution (solution 2), was added. The combined solutions were added to solution 1. The prepared medium (blue to dark green color) was poured into plates and 2 µL of each isolated strain (OD_{600} = 0.6) was spot inoculated onto the medium in duplicates. Plates were incubated at 30˚C for 24 h. Siderophore production was examined by observing an orange halo around colonies (Husen, 2003; Pahari et al., 2017). This experiment was repeated three times, independently.

2.2.14 Cellulase production

The medium used for this test was the same as for lipase production, except that instead of tween-20, the medium was supplemented with 1% carboxymethyl cellulose (CMC). Two µL of each isolated strain (OD_{600} = 0.6) (*E. coli* = negative control) was spot inoculated onto the medium in duplicate. Plates were incubated at 28˚C for 3-4 d. Cellulase production was detected through staining and de-staining with 0.1% Congo Red (w/v in distilled water) for 15 min and 1M NaCl for 15 min, respectively (Kavamura et al., 2013). This experiment was repeated three times, independently.

2.2.15 Ammonia (NH3) production

A colorimetric assay was done following the methods of (Kavamura et al., 2013) with minor modifications. Freshly grown bacterial isolates were inoculated in 10 mL peptone water (15 g peptone water in 1000 mL distilled water) and incubated at 28˚C on a shaker (200 rpm) for 48 h. After incubation, 1 mL of each bacterial sample was transferred to a 1.5 mL tube and centrifuged for 10 minutes (2,218 x g) in a microfuge. The supernatant was transferred to another 1.5 mL tube and 100 µL Nessler's reagent [10% HgI2; 7% KI; 50% aqueous solution of NaOH (32%)] was added to each tube and incubated at room temperature for 30 min. Samples with containing ammonia turned a yellow to orange color. Optical density of each sample was measured in a UV-spectrophotometer at 520 nm. A standard curve (with concentrations of 0-300 µg mL^{-1} using ammonia carbonate) (Appendix C.) was created for calculating the µg of NH_3 per mL of the mixture solution (Kavamura et al., 2013). This experiment was repeated three times, independently.

2.2.16 *In vitro* assay for salt tolerance

Bacterial isolates were spot inoculated in three replicates on lysogeny broth (LB) medium (tryptone 10 g L^{-1}, NaCl 10 g L^{-1}, yeast extract 5 g L^{-1}, agar 15 g L^{-1}) supplemented with 0%, 2%, 4%, 6% and 8% NaCl. LB plates were incubated at 28˚C for 5 d. Plates were examined for bacterial growth on LB with different NaCl concentration (Shikha Gupta & Pandey, 2019). This experiment was repeated three times, independently.

2.2.17 Whole genome sequencing of 5 candidate PSB

Five PSB isolates from the Carolino 164 (*indica*) endosphere, Carolino 164 phyllosphere, and Krasnodarskij 3352 (*japonica*) phyllosphere were chosen based on differing colony morphologies and their ability to produce plant hormones for whole genome sequencing. Nucleic acid extraction of the 5 PSB was done using the Qiagen DNeasy Blood and Tissue Extraction kit according to the manufacturer's instructions. The DNA concentration of each sample was measured with a Nanodrop spectrophotometer. The PSB samples were then sent to the Microbial Genome Sequencing center (www.migscenter.com) for whole genome sequencing. Libraries were prepared as described previously (Baym et al., 2015) and sequenced on an Illumina NextSeq 550 yielding 151-bp paired end reads.

2.2.18 Sequence analysis and annotation of sequenced PSB

Genome sequences of 5 PSB isolates were analyzed using KBase and data were deposited into the public narrative site https://narrative.kbase.us/narrative/52526. Sequence reads were quality checked with FastQC v0.11.9 (Andrews, 2010). Genomes were assembled with SPAdes v3.13.0 (Bankevich et al., 2012) and annotated with RASTtk (Brettin et al., 2015). Initial genome relatedness was determined by inserting a genome into a phylogenetic tree with FastTree2 (Price et al., 2010) and calculating average nucleotide identity with FastANI (Jain et al., 2018) and JSpeciesW (Richter et al., 2016). Taxonomic classification was done with the genome taxonomy database toolkit v1.6.0 (GTDB-tk) (M. D. Lee, 2019). A final maximum likelihood tree was generated using the GToTree v1.6.11 implementation of IQ-TREE2 v2.1.4 (Nguyen et al., 2015) and its dependencies (Edgar, 2004; Capella-Gutiérrez et al., 2009; Hyatt et al., 2010; Eddy, 2011; Tange, 2018; Shen & Xiong, 2019).

2.2.19 Phenotypic fingerprint of sequenced PSB

This test is specialized for characterizing bacteria phenotypically using the Biolog Gen III Microplate. The 5 sequenced PSB were characterized utilizing 71 carbon sources and 23 chemical sensitivity assays on 96 well microplates (Figure 2.1). Isolated bacteria were grown on NA medium and suspended in inoculating fluid (provided with the 96 well microplates) using sterile swabs. The turbidity of inoculating fluid (IF) was 95%. Then, 100 µL of the bacterial suspension were added to each well and microplates were incubated at 28˚C for 2-3 d. Wells that were positive for a specific carbon source turned purple due to formazan (if absorbance determined in a microplate reader exceeded the control well) or were sensitive to a chemical material (if formazan absorbance was less than the control well) (www.biolog.com). This experiment was repeated three times.

GEN III MicroPlate

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2.1 Layout of 71 carbon sources and 23 chemical sensitivity assays (shaded) on the 96 well microplate.

2.2.20 Antibacterial activity of the sequenced PSB against each other

Two culture based antibacterial activity assays, overlay-agar, and cross streak, were done to determine whether the 5 sequenced PSB interact with each other:

Overlay-Agar assay

Isolated bacteria were grown in nutrient broth (NB) media at 30˚C for 24 h. Freshly grown bacteria (OD_{600} = 0.6), were spot inoculated on NA media and incubated at 30˚C for 48 h. After 2 d of incubation, plates were inverted under the hood over filter papers soaked in chloroform for 15 min. Plates were put upright without lids to let extra chloroform evaporate. 10 mL of NB media (containing 1.5% agar at 50˚C) mixed with 200 µL of an indicator strain (the organism to be tested for susceptibility) was poured on the surface of NBA plates and incubated at 30˚C for 48-72 h. After incubation, inoculated bacteria were checked for zones of inhibition (Hockett & Baltrus, 2017). This assay was repeated three times, independently.

Cross-streak agar assay

Isolated bacteria were grown in NB media at 30˚C for 24 h. Freshly grown bacteria (OD_{600} = 0.6) were inoculated in a single 1 cm wide linear streak down the center of NA plates and this process was repeated for each bacterium. Plates were incubated at 30˚C for 48 h. The organism to be tested for susceptibility, was streaked in lines perpendicular towards the bacterium that has been horizontally streaked (up to 1 mm). Plates were incubated at 30˚C for 48-72 h. After incubation, plates were examined for zones of inhibition around the initial bacterium streak, and the width of each zone of inhibition was measured in mm (Carvajal, 1947). This assay was repeated three times, independently.

2.2.21 Evaluating the influence of sequenced PSB on rice growth and development

The influence of five sequenced PSB on rice growth and development was evaluated by seed inoculation with bacterial suspensions [control plants: seeds inoculated in bacterial free KCl]. Shoot and root lengths were measured when plants were 8, 12 and 14 days old. Furthermore, when plants were two-week old, dry weight root/shoot ratio was determined to measure nutrient uptake ability (the higher the root/shoot ratio, the more nutrients are taken up by plants) (Bláha, 2019).

Shoot and root length

Bacterial cells were harvested by centrifugation and rinsed with sterile water, then resuspended in 0.85% KCl (biological saline) (Ma et al., 2009). Carolino 164 and Kasalath (two rice accessions representing *indica* subspecies) as well as Krasnodarskij 3352 and Zhonghua 11 (two rice accessions

representing *japonica* subspecies) seeds were surface sterilized as described earlier and soaked in the bacterial suspension (10^8 cell mL^{-1}) overnight (Gholamalizadeh et al., 2017). Soaked seeds in KCl solution were used as controls and counted as non-bacterial soaked seeds. Both soaked and non-bacterial soaked seeds were germinated in 1X MS media at 30˚C for two days. Germinated seeds were transferred to autoclaved PCR strips placed in boxes filled with sterile water to grow hydroponically. The boxes were put in controlled chambers [12 h of light (28˚C) and 12 h of dark cycle (24˚C)] for 14 days). At day 10, water in boxes were replaced with ¼ Murashige and Skoog (MS) medium. Shoot and root lengths were measured when plants were 14 days old.

To confirm that bacteria were attached to the seeds during overnight shaking (the process of bacterial inoculation) and that uninoculated soaked seeds were free of bacteria, 5 rice seeds from each of both treatments (KCl or bacterial suspension in KCl) were placed onto Nutrient Agar (NA) plates and incubated at 30˚C overnight. Inoculated seeds had bacterial colonies around them, while uninoculated seeds showed no growth of bacteria or contamination (Figure 2.2). In addition, three inoculated and uninoculated seeds were transferred into 1 mL phosphate buffer solution (PBS) separately (1 seed per tube), then tubes were sonicated in a water bath for 10 minutes (Branson, 50-60 Hz). Serial dilution of the sonicated PBS containing seeds were prepared (from 10^{-1} to 10^{-5}) and 10 µL of each dilution were dropped onto NA in triplicate. Plates were incubated at 30˚C overnight. No growth was observed from tubes containing uninoculated seeds. From tubes containing inoculated seeds, the mean Colony Forming Units (CFU) per seed was calculated. The average CFU per seed for Kasnodarskij 3352 and Zhonghua 11 were: 1.13×10^4 and 2.03×10^4, respectively. The average CFU per seed for Carolino 164 and Kasalath were: 1.56×10^4 and 1.78×10^4, respectively (Figure 2.2).

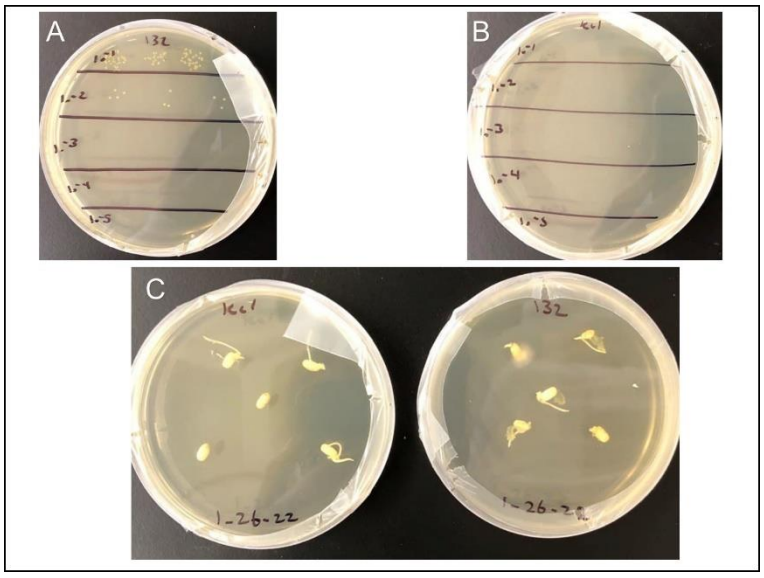

Figure 2.2A. Bacterial colonies from 10 µL drops (triplicates) of serial dilutions of 1 mL PBS containing 1 inoculated seed after sonication in three replicates. This experiment was done twice independently. The average CFU per seed for *japonica* was: 1.13 x 10^4. The average CFU per seed for *indica* was: 1.56 x 10^4. **B.** No bacterial growth from seeds soaked in sterile KCl. **C.** 5 *japonica* seeds on NA medium after overnight shaking in KCl containing 10^8 bacterial cells [right, labelled as 132 (code for one bacterial isolate)] or cell-free KCl [left, labelled as KCl (bacterial free solution)]. Bacterial growth from soaked seeds in bacterial suspension can be seen, while no bacterial growth was observed in seeds soaked in cell-free KCl.

Dry weight root/shoot ratio

Shoots and roots of 15 inoculated and uninoculated plants (two-week old plants) were isolated and put into a dry oven at 50˚C temperature for 48 h (Gholamalizadeh et al., 2017). After two days of incubation, the dry weight root/shoot ratio of each of 15 plants per trial (total of 3 trials) were measured to evaluate the ability of plants to take up nutrients while they were inoculated with bacterium.

2.2.22 Data availability

All sequence reads were deposited into the NCBI Sequence Read Archive (SRA) under BioProject PRJNA667792 and SRA accessions SRS7484588 - SRS7484592. All assemblies and annotations can be viewed, manipulated, and downloaded from the open access KBase narrative https://narrative.kbase.us/narrative/52526. We have also created a static narrative to support the manuscript and can be accessed here: https://kbase.us/n/52526/166/.

2.2.23 Statistical analysis

All statistical comparisons (ANOVA and Tukey post-hoc) among the 25 PSB was done using Minitab® Statistical Software, Version 20.2.0.0 (free access from University of Wisconsin Milwaukee).

Comparisons (Kruskal–Wallis, Dunn post-hoc) between shoot and root lengths or dry weight root to shoot ratio was done using R Software v4.0.4. Kruskal-Wallis and Dunnett post-hoc test were implemented using the rstatix R package.

2.3 Results

2.3.1 Bacterial isolation and characterization

To identify plant growth promoting bacteria (PGPB) associated with rice plants, a total of 140 bacteria were isolated and purified from the phyllosphere, root endosphere, and root rhizoplane of two accessions representing the two *japonica* and *indica* subspecies of rice (Table 2.3). The colony morphology and Gram reaction of each isolated bacterium are listed in Appendix D. Because phosphate is the second most important macronutrient for plant growth (Nosrati et al., 2014), we reasoned that mineral phosphate solubilizing bacteria (PSB) associated with rice tissues might act as PGPB. Phosphate is mostly insoluble in the soil and therefore unavailable to plants (Nautiyal, 1999). There are PSB in the soil with the ability of providing soluble phosphate through releasing organic acids and acid phosphatase (Oteino et al., 2015). To identify PSB among the 140 isolates, bacteria were spotted onto agar plates containing insoluble phosphate ($Ca_3(PO_4)_2$). Of the 140 isolates, 25 (18%) were able to solubilize mineral phosphate as shown by a halo around the bacterial colonies. The mean phosphate solubilization index was calculated for those 25 bacteria. There was no significant difference in the solubilization index of all 25 PSB ($p > 0.05$, ANOVA) (Figure 2.3A).

Table 2.3 Number of bacterial isolates from two accessions, Krasnodarskij 3352 and Carolino 164, representing the *japonica* and *indica* subspecies of rice (*Oryza sativa* L.), respectively.

Rice subspecies/ plant tissue compartment	Root rhizoplane	Root endosphere	Phyllosphere
japonica	36	30	17
indica	20	22	15

We next characterized these 25 phosphate solubilizing isolates for other possible plant growth promoting traits, including nutrient sequestration and hormone production (Yadav et al., 2017). Seventeen of the 25 (68%) PSB isolates were considered nitrogen fixing microorganisms that could also contribute to plant growth (Figure 2.4E and Table 2.4). Only one isolate (n00078) was able to solubilize zinc sulfate (Figure 2.4D). Of the phytohormones tested, all 25 PSB isolates had the ability of indoleacetic acid (IAA) production, and among them, isolate n00132 produced the highest amount of IAA (10.8 µg mL^{-1}; $p < 0.05$,

ANOVA, Tukey post-hoc), two to three times more than other isolates (Figure 2.3B). Gibberellic acid production was observed in 72% (18/25) of the isolates (Figure 2.3C). There was no difference in GA producation among the 18 bacteria ($p > 0.05$, ANOVA).

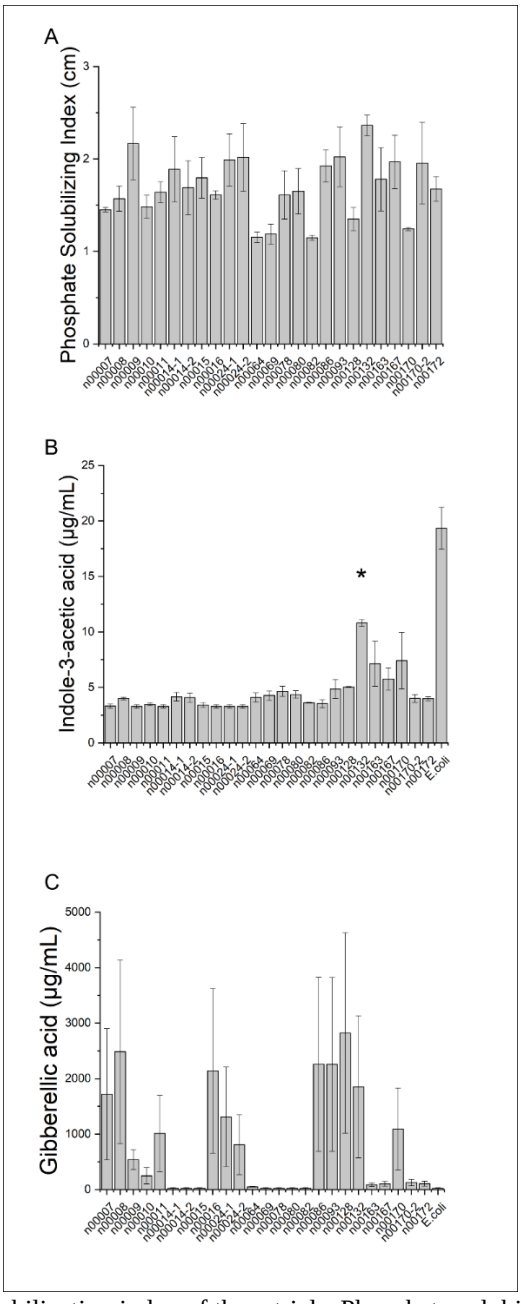

Figure 2.3A. Mean phosphate solubilization index of three trials. Phosphate solubilization activity was evaluated by the measurement of halo diameters around bacterial colonies (in cm) relative to colony diameter on NBRIP (National Botanical Research Institute's Phosphate) medium. There was no significant difference in the solubilization index of all 25 PSB ($p > 0.05$, ANOVA). **B.** Indoleacetic acid (IAA) production by phosphate solubilizing bacteria (PSB). *E. coli* strain OP50 was used as positive control for IAA production. n00132 produced a significantly higher amount of IAA than other PSB isolates, except for

n00170 ($p < 0.05$, ANOVA and Tukey post-hoc test, E. coli was not used in statistical analysis). **C.** Gibberellic acid (GA) production by phosphate solubilizing bacteria (PSB). *E. coli* strain OP50 used as negative control for GA production. PSB isolates n00014-1, n00014-2, n00015, n00064, n00069, n00078 and n00080 were not able to produce GA as they were not different from the negative control. There was no difference in GA production among the 18 bacteria ($p > 0.05$, ANOVA). Error bars show standard errors.

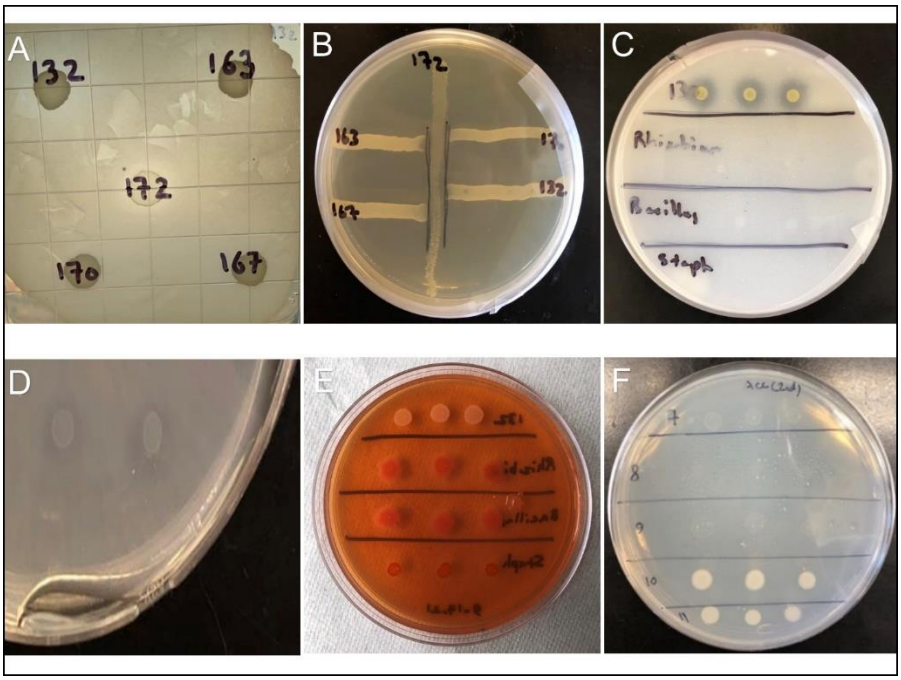

Figure 2.4A. Overlay agar assay. Halo around 172 (*Paenibacillus graminis*), showing growth inhibition when 132 (*Pseudomonas mosselii*) is overlaid. Halo around 172 was also observed, when the other three phosphate solubilizing bacteria (PSB) were overlaid: 163, *Paenibacillus rigui*; 167, *Brevibacillus* sp900114075; 170, *Microvirga* sp003151255. **B.** Cross-streak assay. Growth inhibition of the other four PSB is shown when 172 is in the middle of the NB plate. **C.** Bacterium 132 isolated from the *indica* phyllosphere has high phosphate solubilization ability as shown by strong halo formations (2.36 cm) around the bacterial colonies. *Rhizobacterium* sp., *Bacillus* sp., and *Staphylococcus aureus* are negative controls. **D.** Bacterium n00078 isolated from *japonica* root endosphere has zinc solubilization ability as shown by halo formation around the bacterial colony. **E.** Bacteria with nitrogen fixation ability shown on CR-YMA plates. The lower the absorption of Congo Red dye, the higher the ability for nitrogen fixation. In this figure, one strong nitrogen fixing bacterium (top 3 colonies; *P. mosselii*) is along with *Rhizobacterium* sp. and *Bacillus* sp., as positive controls and *Staphylococcus aureus* as a negative control. **F.** Phosphate solubilizing bacteria n00007 (7), n00008 (8), n00009 (9), n00010 (10), and n00011 (11) are shown with the ability of ACC deaminase production based on their growth on DF minimal salt medium supplemented with 3 mM ACC as sole nitrogen source.

Table 2.4 Phosphate solubilizing bacteria with nitrogen fixation ability on CR-YMA plates. ++ (Pale white-pink), + (White-pink), - (Strong pink-red). Bacterial colonies with colors ranging from white to pale white-pink were able to fix nitrogen due to cleavage of the azo bond (-N=N-) in Congo Red.

PSB codes	**Colony color**
n00007	-
n00008	-
n00009	-

n00010	+
n00011	++
n00014-1	++
n00014-2	++
n00015	++
n00016	++
n00024-1	++
n00024-2	++
n00064	-
n00069	-
n00078	-
n00080	-
n00082	-
n00086	+
n00093	++
n00128	++
n00132	++
n00163	++
n00167	++
n00170	++
n00170-2	++
n00172	+
Rhizobium sp.	++
Bacillus sp.	++
Staphylococcus aureus	-

High soil salinity negatively affects the ability of plant roots to take up water and bacteria that can tolerate high concentrations of NaCl were previously shown to protect plants from water deficiency stress (Mehnaz et al., 2010; Numan et al., 2018). Therefore, all 25 PSB isolates were tested for salt tolerance on LB medium with different concentrations of NaCl (2%, 4%, 6% and 8%). All 25 PSB were able to tolerate at least 2% NaCl and 80% (20/25) of them grew on LB with 4% NaCl (Table 2.5). Only one bacterium (n00014-1) grew on LB with 6% NaCl and none of the PSB were able to tolerate 8% NaCl.

Table 2.5 Indirect plant growth promoting assays and *in vitro* assay results for phosphate solubilizing bacteria (PSB) salt tolerance. "+" results are shaded.

PSB codes	Siderophore	Lipase	Cellulase	Antifungal activity (%)	Gelatin	Casein	Ammonia (µg/mL)	LB (2%) NaCl	LB (4%) NaCl	LB (6%) NaCl	LB (8%) NaCl
n00007	+	+	-	23.3	+	-	780.24	+	+	-	-
n00008	-	+	+	4.43	+	-	811.58	+	+	-	-
n00009	+	+	-	25.14	+	-	850.20	+	+	-	-
n00010	+	+	-	20.9	-	-	805.08	+	+	-	-
n00011	+	+	+	21.82	+	+	929.22	+	+	-	-
n00014-1	+	+	+	22.67	Slight +	-	895.55	+	+	+	-
n00014-2	+	+	+	19.76	-	-	894.48	+	-	-	-
n00015	+	+	+	25.74	+	-	883.17	+	-	-	-
n00016	+	+	+	11.17	-	-	859.05	+	-	-	-
n00024-1	+	+	+	14.98	-	-	871.64	+	-	-	-
n00024-2	+	+	+	18.88	-	-	835.04	+	-	-	-
n00064	+	+	-	7.06	+	-	828.38	+	+	-	-
n00069	+	-	Slight +	8.77	-	-	858.33	+	+	-	-
n00078	-	-	Slight +	8.19	+	-	891.15	+	+	-	-
n00080	-	-	Slight +	2.68	Slight +	-	862.91	+	+	-	-
n00082	-	-	Slight +	0	Slight +	-	850.93	+	+	-	-
n00086	-	-	Slight +	0	-	-	840.47	+	+	-	-
n00093	-	-	Slight +	1.7543	-	-	876.94	+	+	-	-
n00128	+	+	+	23.73	+	-	846.75	+	+	-	-
n00132	+	+	+	5.75	+	+	811.10	+	+	-	-
n00163	+	+	+	0	+	-	803.43	+	+	-	-
n00167	+	+	+	3.95	Slight +	-	845.71	+	+	-	-
n00170	-	-	Slight +	3.74	-	-	823.14	+	+	-	-
n00170-2	+	+	+	22	-	-	856.44	+	+	-	-
n00172	-	-	Slight +	0	-	-	807.13	+	+	-	-

Ethylene is a phytohormone that plant roots produce for developmental processes such as xylem formation and to regulate stress responses (Glick et al., 2007). When plants are under stress, they often emit excessive ethylene, which has a negative effect on plant development. Bacteria that produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase convert ACC, a rate limiting precursor for ethylene production, to ammonia and α-ketobutyrate, which can protect plants from excessive amounts of ethylene (Ozimek et al., 2018). In this study, PSB that produced ACC deaminase enzyme were examined based on their ability to grow on DF minimal salt medium supplemented with 3 mM ACC as sole nitrogen

source (Figure 2.3F and Table 2.7). 84% (21/25) of the isolates had ACC deaminase activity that might protect plants from excessive ethylene buildup (Table 2.6).

Table 2.6 Phosphate solubilizing bacteria (PSB) with ACC deaminase production. "Strong +" indicates PSB grew strongly on DF minimal salt medium supplemented with 3 mM ACC as sole nitrogen source, while "Slight +" indicates weak growth. "None" means no growth.

PSB codes	**ACC deaminase (overall 3 trials)**
n00007	Slight +
n00008	None
n00009	Slight +
n00010	Strong +
n00011	Strong +
n00014-1	Strong +
n00014-2	Strong +
n00015	Strong +
n00016	Strong +
n00024-1	Strong +
n00024-2	Strong +
n00064	Slight +
n00069	Slight +
n00078	None
n00080	None
n00082	Slight +
n00086	Slight +
n00093	None
n00128	Strong +
n00132	Slight +
n00170	Slight +
n00170-2	Strong +
n00172	Slight +
n00163	Slight +
n00167	Slight +
E. coli strain OP50	None
Agrobacterium	Strong +
Serratia marcescens	Slight +

PSB isolates were next tested for antifungal activity, lipase production, protease activity (casein and gelatin hydrolyzing), cellulase and ammonia production, all of which can contribute to bacterial defense mechanisms against plant pathogens (Dinesh et al., 2015). PSB isolates were also tested for

siderophore production, which is a low molecular mass compound with high affinity to Fe^{3+} and facilitates iron uptake by plants (M. S. Khan et al., 2014). Results for these indirect PGPB assays are summarized in Table 2.5. Based on the results, 68% (17/25) of the PSB isolates were able to facilitate iron uptake in plants and produce lipase enzyme, and 84% (21/25) of phosphate solubilizing bacteria were able to produce cellulase enzymes. 56% (14/25) of the phosphate solubilizing isolates were able to liquify gelatin, among which 71% (10/14) strongly liquified it and 29% (4/14) slightly liquified it. Only 8% (2/25) of the phosphate solubilizing isolates were able to hydrolyze casein. 84% (21/25) of the PSB isolates were able to inhibit fungal pathogen growth. All 25 PSB isolates were able to produce ammonia. These results showed that all 25 bacterial isolates tested had some ability to indirectly improve plant growth through pathogen control.

2.3.2 Whole genome sequencing and phenotypic analysis of five phosphate solubilizing bacteria

Five bacterial isolates that were positive for phosphate solubilization, IAA and gibberellic acid production, and for nitrogen fixation were selected for whole genome sequencing. Genome assembly statistics and taxonomic assignments for the five isolates are shown in Table 2.8. Three of the isolates (n00163, n00167, n00172) were members of the Gram-positive *Paenibacillaceae* family, while the others belonged to the Gram-negative Proteobacteria phylum (Figure 2.5). Three of the five isolates (n00163, n00167, n00170) fell near or below the 95% average nucleotide identity (ANI) threshold that is sometimes used for species delineation (Richter et al., 2016; Olm et al., 2020; Rodriguez-R et al., 2021), indicating that these isolates are potentially new species. The other two isolates were new strains of *Pseudomonas mosselii* and *Paenibacillus graminis* (Table 2.7). All five of the isolates had genes predicted for synthesis of the phytohormone auxin (IAA), for ammonia assimilation, and for phosphate metabolism. These genome annotations provide the genotypes to accompany their plant growth promoting phenotypes.

Table 2.7 Feature types and assemble Reads with SPAdes - v3.13.0 results (QUAST analysis) of sequenced genomes and genome Taxonomy Database (GTDB) analysis results of phosphate solubilizing bacteria isolated from different rice subspecies and tissues.

	n00163	**n00167**	**n00132**	**n00172**	**n00170**
Genome size	7,304,039	6,449,936	5,782,008	7,161,689	4,587,927
G+C ratio	52.38	49.37	64.3	50.42	63
# of contigs	89	153	133	104	46
N50	172,653	100,283	76,736	161,901	235,294
Coverage predicted genes	6856	6759	5305	6966	4557
Rice subspecies & tissue compartment	*indica* endosphere	*indica* leaves	*indica* leaves	*japonica* leaves	*japonica* leaves
Average nucleotide identity(%)	80.61	95.39	99.23	98.41	82.89
Closest genome	*Paenibacillus rigui*	*Brevibacillus sp900114075*	*Pseudomonas mosselii*	*Paenibacillus graminis*	*Microvirga sp003151255*

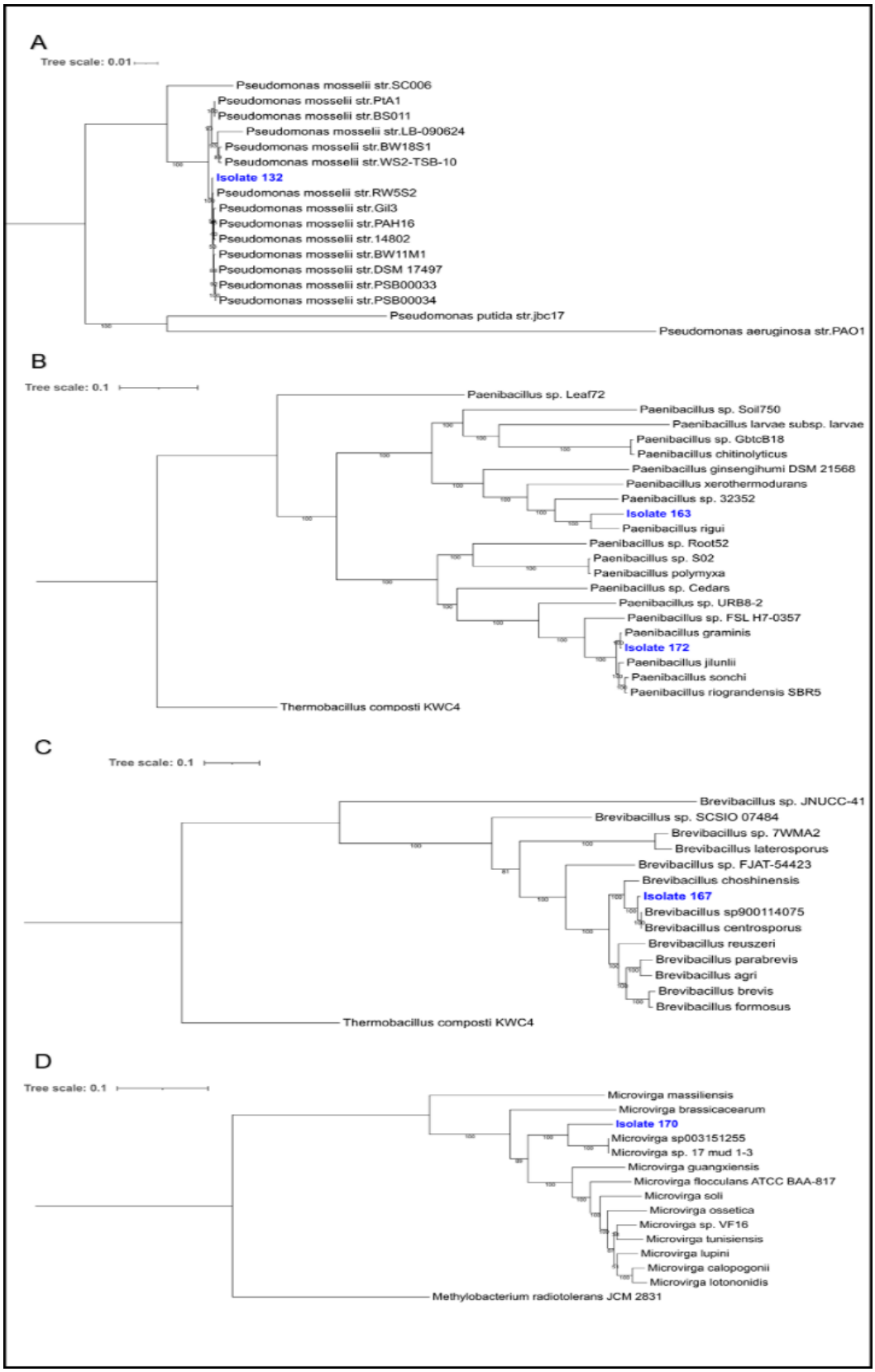
A
Tree scale: 0.01
Pseudomonas mosselii str.SC006
Pseudomonas mosselii str.PtA1
Pseudomonas mosselii str.BS011
Pseudomonas mosselii str.LB-090624
Pseudomonas mosselii str.BW18S1
Pseudomonas mosselii str.WS2-TSB-10
Isolate 132
Pseudomonas mosselii str.RW5S2
Pseudomonas mosselii str.Gil3
Pseudomonas mosselii str.PAH16
Pseudomonas mosselii str.14802
Pseudomonas mosselii str.BW11M1
Pseudomonas mosselii str.DSM 17497
Pseudomonas mosselii str.PSB00033
Pseudomonas mosselii str.PSB00034
Pseudomonas putida str.jbc17
Pseudomonas aeruginosa str.PAO1
B
Tree scale: 0.1
Paenibacillus sp. Leaf72
Paenibacillus sp. Soil750
Paenibacillus larvae subsp. larvae
Paenibacillus sp. GbtcB18
Paenibacillus chitinolyticus
Paenibacillus ginsengihumi DSM 21568
Paenibacillus xerothermodurans
Paenibacillus sp. 32352
Isolate 163
Paenibacillus rigui
Paenibacillus sp. Root52
Paenibacillus sp. S02
Paenibacillus polymyxa
Paenibacillus sp. Cedars
Paenibacillus sp. URB8-2
Paenibacillus sp. FSL H7-0357
Paenibacillus graminis
Isolate 172
Paenibacillus jilunlii
Paenibacillus sonchi
Paenibacillus riograndensis SBR5
Thermobacillus composti KWC4
C
Tree scale: 0.1
Brevibacillus sp. JNUCC-41
Brevibacillus sp. SCSIO 07484
Brevibacillus sp. 7WMA2
Brevibacillus laterosporus
Brevibacillus sp. FJAT-54423
Brevibacillus choshinensis
Isolate 167
Brevibacillus sp900114075
Brevibacillus centrosporus
Brevibacillus reuszeri
Brevibacillus parabrevis
Brevibacillus agri
Brevibacillus brevis
Brevibacillus formosus
Thermobacillus composti KWC4
D
Tree scale: 0.1
Microvirga massiliensis
Microvirga brassicacearum
Isolate 170
Microvirga sp003151255
Microvirga sp. 17 mud 1-3
Microvirga guangxiensis
Microvirga flocculans ATCC BAA-817
Microvirga soli
Microvirga ossetica
Microvirga sp. VF16
Microvirga tunisiensis
Microvirga lupini
Microvirga calopogonii
Microvirga lotononidis
Methylobacterium radiotolerans JCM 2831

Figure 2.5 Maximum likelihood phylogenetic trees based on whole genome sequences of five bacteria isolated from different rice tissue compartments. The tree scale is the number of nucleotide substitutions per site. **A.** *Pseudomonas mosselii*, isolate 132 from *indica* leaves. **B.** *Paenibacillus rigui*, isolate 163 from *indica* endosphere and *Paenibacillus graminis*, isolate 172 from *japonica* leaves. **C.** *Brevibacillus* sp900114075, isolate 167 from indica leaves. **D.** *Microvirga* sp003151255, isolated 170 from *japonica* leaves.

Gen III microplates (Biolog) were used to characterize the metabolic phenotypes of the five sequenced PSB. *Agrobacterium tumefaciens*, a Gram-negative plant pathogen producing crown gall tumors (W. Li et al., 2020), was used as a comparison because of its well-characterized ability to use plant metabolites as nutrients (Subramoni et al., 2014). Of the five isolates tested, two (n00163 and n00167) shared similar phenotypic carbon source usage characteristics (Table 2.8). Of the different metabolic phenotypes, methyl pyruvate is a plant chemoeffector (Feng et al., 2021) and glycerol is a leaf exudate as a result of carbon dioxide fixation in photosynthesis (Gerber et al., 1988). Glycerol was not used by n00132, while methyl pyruvate was not used by n00132 and n00172. Interestingly, n00172 was the only bacterium able to utilize stachyose (raffinose family of oligosaccharides in plants) (Sengupta et al., 2015). All five sequenced PSB isolates shared the same phenotypic chemical resistance against pH 6, 1% NaCl, 1% sodium lactate, guanidine HCl, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite and aztreonam. Only one bacterium (n00172) showed resistance against Rifamycin SV and sodium butyrate (Table 2.9).

Table 2.8 Bacterial phenotypic fingerprint of carbon source usage (Biolog microplates). "+" (shaded area) indicates bacterial use of the specific carbon source and "-" indicates that bacteria did not grow in the microplate. n00132, *Pseudomonas mosselii*; n00163, *Paenibacillus rigui*; n00167, *Brevibacillus sp900114075*; n00170, *Microvirga sp003151255*; n00172, *Paenibacillus graminis.*

Carbon Sources	*Agro*	n00132	n00163	n00167	n00170	n00172
Dextrin	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
D-Turanose	+	+	+	+	+	+
Stachyose	+	-	-	-	-	+
D-Raffinose	+	-	-	-	-	-
α-D-Lactose	+	-	-	-	+	+
D-Melibiose	+	-	-	-	-	+
ß-Methyl-D-Glucoside	+	+	+	+	+	+

D-Salicin	+	+	+	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+	+
N-Acetyl-ß-D-Mannosamine	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
D-Fucose	+	-	-	-	-	-
L-Fucose	+	-	-	-	-	-
L-Rhamnose	+	-	-	-	-	-
Inosine	+	+	+	+	+	+
D-Sorbitol	+					
D-Mannitol	+	+	+	+	+	+
D-Arabitol	+	-	-	-	-	-
myo-Inositol	+	-	-	-	-	-
Glycerol	+	-	+	+	+	+
D-Glucose-6-PO_4	+	-	-	-	-	-
D-Fructose-6-PO_4	+	-	-	-	-	-
D-Aspartic Acid	+	-	-	-	-	-
L-Alanine	+	-	-	-	-	-
L-Aspartic Acid	+	-	-	-	-	-
L-Glutamic Acid	+	-	-	-	-	-
L-Pyroglutamic Acid	+	-	-	-	-	-
L-Serine	+	-	-	-	-	-
Pectin	+	+	+	+	+	+
D-Galacturonic Acid	+	-	-	-	-	-
L-Galactonic Acid Lactone	+	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+	+
D-Glucuronic Acid	+	-	-	-	-	-
Glucuronamide	+	-	-	-	-	-
Mucic Acid	+	-	-	-	-	-
Quinic Acid	+	-	-	-	-	-
Methyl Pyruvate	+	-	+	+	+	-
D-Lactic Acid Methyl Ester	+	-	-	-	-	-
L-Lactic Acid	+	+	+	+	+	+
D-Malic Acid	+	-	-	-	-	-
L-Malic Acid	+	-	-	-	-	-
Bromo-Succinic Acid	+	-	-	-	-	-
Tween 40	+	+	+	+	+	+
Acetoacetic Acid	+	+	+	+	+	
Propionic Acid	+	-	-	-	-	-
Acetic Acid	+	-	-	-	-	-

Table 2.9 Bacterial phenotypic fingerprint of chemical sensitivity (Biolog microplates). "+" (shaded area) indicates bacterial resistance to a specific chemical and "-" indicates bacterial sensitivity to a specific chemical.

Chemical inhibitors resistance	*Agro*	n00132	n00163	n00167	n00170	n00172
pH 6	+	+	+	+	+	-
1% NaCl	+	+	+	+	+	+
1% Sodium Lactate	+	+	+	+	+	+
Troleandomycin	+	-	-	-	-	-
Rifamycin SV	+	-	-	-	-	+
Lincomycin	+	-	-	-	-	
Guanidine HCl	-	+	+	+	+	+
Tetrazolium Blue	-	-	-	-	-	-
Nalidixic Acid	-	+	+	+	+	-
Lithium Chloride	-	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+	-
Aztreonam	+	+	+	+	+	-
Sodium Butyrate	-	-	-	-	-	+

2.3.3 Antibacterial activity of the sequenced PSB against each other

Overlay-agar and cross streak assays were done to determine whether the five sequenced PSB were compatible with each other. Plants derive greater benefits from the mixture (consortium) of PGPB than one bacterium (Molina-Romero et al., 2017). Therefore, it is important to investigate whether these five PSB would compete, act neutrally, or synergistically to improve rice growth. Based on these assays, only isolate n00172 competitively inhibited the growth of the other four PSB (Figure 2.3A & B). This indicates that n00172 (*Paenibacillus graminis*) produced inhibitory chemicals that did not allow other bacteria to grow in its close proximity (Table 2.10). Analysis of n00172 genome through the program antiSMASH (Blin et al., 2021) revealed this bacterium has several biosynthetic gene clusters that are predicted to have antibiotic activity (Table 2.11) that may be responsible for competitive inhibition of other species.

Table 2.10 Growth inhibition by n00172 (*Paenibacillus graminis*). Two antibacterial assays, overlay-agar and cross streak, show n00172's ability to inhibit the growth of the four other phosphate solubilizing bacteria (PSB). In overlay-agar test, "+" (shaded area) indicates presence of an inhibition halo around n00172 when each of these four PSB are overlaid. In the cross-streak test, growth inhibition of the four other PSB when crossing the n00172 bacterium is shown in mm (the test was done twice). n00132, *Pseudomonas mosselii*; n00163, *Paenibacillus rigui*; n00167, *Brevibacillus sp900114075*; n00170, *Microvirga sp003151255*.

Region	Type	From	To	Most similar known cluster	Similarity
3.1	NPRS, T1PKS, NRPS-like	181,635	238,230		
4.1	cyclic-lactone-autoinducer	812	21,351		
4.2	RRE-containing	55,265	76,665		
5.1	cyclic-lactone-autoinducer	34,965	55,683		
7.1	NPRS, T1PKS	1	38,457	xenocoumacin 1 / xenocoumacin II, NRP + Polyketide:Modular type I	28%
10.1	NPRS, transAT-PKS	154,535	193,126		
13.1	cyclic-lactone-autoinducer	150,188	161,901		
20.1	NPRS, transAT-PKS, T1PKS, NRPS-like	1	97,214		
30.1	lassopeptide	17,331	41,158	Paeninodin, RiPP	100%
33.1	RRE-containing	25,743	47,170		
51.1	cyclic-lactone-autoinducer	4,648	25,296		
55.1	cyclic-lactone-autoinducer	16,231	36,755		
69.1	NRPS-like	1	13,082		
71.1	NPRS	1	10,121		
76.1	transAT-PKS-like	1	4,361		
77.1	NPRS	1	2,877		
83.1	NPRS	1	1,397		

Table 2.11 Secondary metabolite regions of isolate n00172 (*Paenibacillus graminins*) identified using strictness 'relaxed'. NRPS = Non-ribosomal peptide synthetase cluster, Type I PKS = Type I Polyketide synthase.

Antibacterial activity/Bacteria	n00132	n00163	n00167	n00170	n00172
Overlay-agar	-	-	-	-	+
Cross-streak	2 mm/1 mm	3 mm/1 mm	1 mm/1 mm	1 mm/1 mm	-

2.3.4 Influence of sequenced PSB on rice plant growth and development

To determine whether the five sequenced PSB act as PGPB, seeds from the four rice varieties, representing the *indica* and *japonica* subspecies, were inoculated individually with one of the five PSB, and the shoot and root development (length) and biomass (dry weight) of two-week old plants were compared to uninoculated control plants (Figure 2.6). Control plants were from uninoculated seeds that were soaked in KCl solution without any bacteria. *Indica* Kasalath varietal plants inoculated with n00163 (*Paenibacillus rigui*) and n00170 (*Microvirga* sp.) had significantly higher relative shoot growth compared to Kasalath control plants (Figure 2.6A; $p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test). There was no shoot growth promotion observed in *indica* Carolino 164 varietal plants inoculated with the five bacteria. On the other hand, *japonica* Kasnodarskij 3352 varietal plants inoculated with n00132 (*Psudomonas mosselii*) had significantly higher relative shoot growth compared to Kasnodarskij 3352 control plants (Figure 2.6B; $p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test). Similar to *indica* Carolino 164 varietal plants, no shoot growth promotion was observed in *japonica* Zhonghua 11 varietal plants inoculated with the five bacteria. In addition to relative shoot length, *indica* Kasalath and *japonica* Zhonghua 11 varietal plants inoculated with n00132 (*Pseudomonas mosselii*) and n00163 (*Paenibacillus rigui*), respectively, responded negatively in relative root length ratio compared to their control varietal plants (Fig 2.6C&D; $p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test). On the other hand, *japonica* Kasnodarskij 3352 varietal plants inoculated with n00170 (*Microvirga* sp.) and n00172 ((*Paenibacillus graminis*) had significantly increased root growth compared to Kasnodarskij 3352 control plants (Figure 2.6D); $p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test). Taken together, this showed that the four bacterial species n00132 (*Pseudomonas mosselii*), n00163 (*Paenibacillus rigui*), n00170 (*Microvirga* sp.) and n00172 (*Paenibacillus graminins*) were PGPB for the *japonica* Kasnodarskij 3352 and *indica* Kasalath varietal plants for the characteristics tested here, while none of the five species were PGPB for the *japonica* Zhonghua 11 or *indica* Carolino 164 varietal plants of rice.

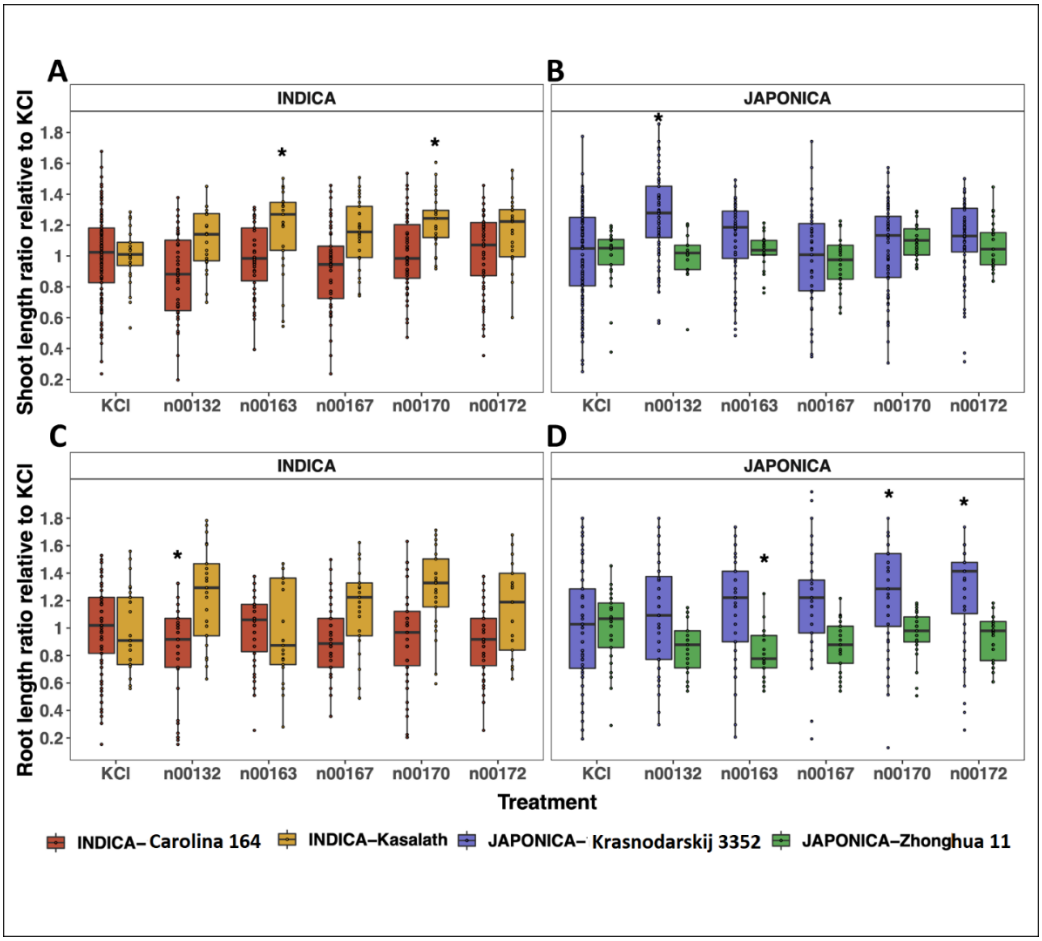

Figure 2.6 Relative shoot (A and B) and root (C and D) growth of the 2-week-old treated plants with five bacterial species compared to shoot and root growth of the uninoculated control plants (KCl). **A & B.** Shoot length ratio relative to control (KCl) plants belong to *indica* and *japonica* subspecies: *indica* Carolina 164, *indica* Kasalath, *japonica* Krasnodarskij 3352 and *japonica* Zhonghua 11. **C & D.** Root length ratio relative to control (KCl) plants belong to *indica* and *japonica* subspecies: indica Carolina 164, *indica* Kasalath, *japonica* Krasnodarskij 3352 and *japonica* Zhonghua 11. Each dot represents an individual plant. Statistical significance was determined by Kruskal–Wallis and Dunnett post-hoc tests. *: $p < 0.05$.

Root/shoot ratio refers to the biomass that is growing underground and reflects the ability of plants to take up nutrients. The rationale for assessing this is that the higher this ratio, the greater the ability of plants to take up nutrients, which correlates with increased stress tolerance (Bláha, 2019). Our measurements showed that *Indica* Carolino 164 inoculated with n00170 (*Microvirga* sp.) and n00172 (*Paenibacillus graminins*) had lower dry weight root/shoot ratios ($p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test), suggesting that these two species do not help with nutrient uptake (Figure 2.6A). Moreover, the *indica* Kasalath and *japonica* Kasnodarskij 3352 varietal plants inoculated with the five bacteria did not have significantly improved root/shoot ratios compared to uninoculated control plants (Figure 2.7A&B). In

contrast, only the *japonica* Zhonghua 11 varietal plants inoculated with n00132 (*Pseudomonas mosselii*) had improved dry weight root/shoot ratio compared to control plants (Figure 2.7B; $p < 0.05$, Kruskal-Wallis, Dunnett post-hoc test). Taken together only *Pseudomonas mosselii* helped the *japonica* Zhonghua 11 varietal plants in nutrient uptake.

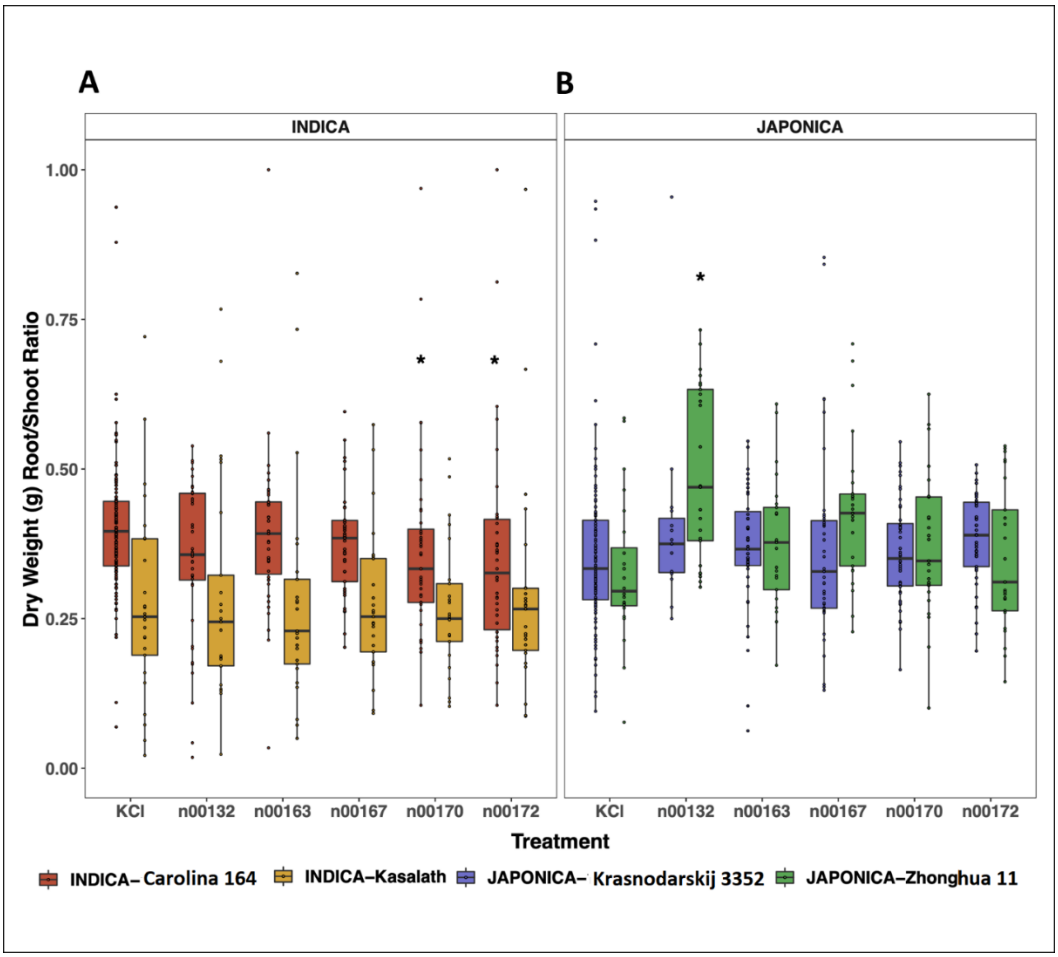

Figure 2.7 Dry weight root/shoot ratios of the 2-week-old treated plants with five bacterial species compared to uninoculated control plants (KCl). **A.** Root/shoot ratios of the two *indica* varieties: *indica* Carolina 164, *indica* Kasalath. **B.** Root/shoot ratios of the two *japonica* varieties: *japonica* Krasnodarskij 3352 and *japonica* Zhonghua 11. Each dot represents an individual plant and outliers (values > 1) were omitted from the plot. Statistical significance was determined by Kruskal–Wallis and Dunnett post-hoc tests. *: $p < 0.05$.

2.4 Discussion

To identify plant growth promoting bacteria (PGPB) from rice, we isolated 140 bacteria residing in or on different tissues of two distantly related rice varieties and screened for phosphate solubilizing activity. It is worth noticing that the Asian rice species (*O. sativa* L.) is divided into two subspecies, long-grain *indica* and short-grain *japonica*, both of which contain several subgroups. The *indica* subspecies contains *aus* and *indica* subgroups, and the *japonica* subspecies contains *tropical japonica*, *temperate japonica*, and *aromatic* subgroups. In this study, bacteria were isolated from *aus* and *temperate japonica* accessions Carolina 164 and Krasnodarskij 3352, respectively, because they represent subgroups with major genotypic and phenotypic differences (Shimoyama et al., 2020). The bacteria isolated from these two varietal groups of rice were tested for their plant growth promoting influence on the rice variety that they were isolated from, and the other three rice varieties listed above.

The vegetative growth stage in rice plants, which encompasses seed germination to stem elongation, is a critical stage for the formation of root exudates (Aulakh et al., 2001) and interactions with bacteria. High amounts and different types of carbohydrates and organic acids are produced by roots at this stage (Aulakh et al., 2001; Zhalnina et al., 2018). Therefore, we chose to work with young seedlings (when rice plants newly developed the second leaf) to observe the interaction between plants and bacteria, as this is when rice plants start to produce different metabolites that can provide bacteria with nutrients.

Of the 140 isolates, 25 were phosphate solubilizing bacteria (PSB) that were also able to produce indole acetic acid, an important plant growth hormone. Based on other direct and indirect PGPB assays, five PSB were selected for whole genome sequencing. The five PSB species were closely related to *Paenibacillus rigui* (n00163, isolated from the Carolino 164 endosphere), *Brevibacillus sp.* (n00167, isolated from Krasnodarskij 3352 leaves), *Pseudomonas mosselii* (n00132, isolated from Carolino 164 leaves), *Paenibacillus graminis* (n00172, isolated from Krasnodarskij 3352 leaves), and *Microvirga sp.* (n00170, isolated from Krasnodarskij 3352 leaves).

Although only four of the PSB improved growth and development of the two *japonica* varieties and *indica* Kasalath variety tested here, all of these bacterial genera were previously shown to function as PGPB (Mano et al., 2007; Torre-ruiz et al., 2016; Grady et al., 2016; Li et al., 2020; Lau et al., 2020). There are some *Brevibacillus* species are able to produce antifungal siderophores (Lau et al., 2020).The

Microvirga genus is a nodule legume endophyte that can improve plant growth by providing ammonia to plants (Jiménez-Gómez et al., 2019). Some species of *Paenibacillus* have been isolated from plant rhizospheres, and *P. graminis* is a known nitrogen fixing bacterium (Estebanez et al., 2006; Rodriguez-R et al., 2021). *P. rigui* is one of the species that has been specifically isolated as an endophyte from *O. sativa* (Raweekul et al., 2016). *Pseudomonas* species are widely present in soils, and it is known that some strains can survive in different environmental conditions and tissues of eukaryotic hosts mostly through producing chemicals that protect them against pathogenic bacteria or fungi (Torre-ruiz et al., 2016; Li et al., 2020). *P. mosselii* was previously shown to be a phosphate solubilizing bacterium (B. K. Jha et al., 2009), and our results are in agreement with this finding (Figure 2.2A). *P. mosselii* is also one of the most common PGPB that inhibits growth of plant pathogens such as *Agrobacterium tumefaciens* (W. Li et al., 2020) and results from our study (cross-streak experiment) confirms this observation (Figure 2.8). In addition, *P. mosselii* is also known for improving plant growth and increasing sugar content of *Agave americana* L. (Torre-ruiz et al., 2016), and our results are in agreement with some of these findings (Figures 2.5 & 2.6). Therefore, this bacterium is a good candidate for promoting plant growth, and potentially also for protecting plants against stress (Karkera et al., 2013).

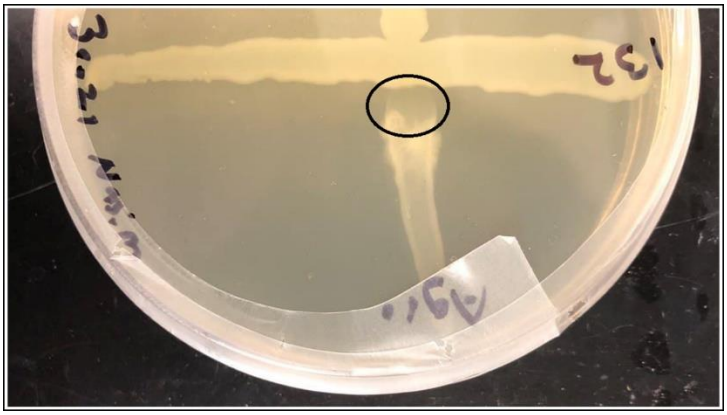

Figure 2.8 Cross-streak assay. Growth inhibition of the *Agrobacterium tumefaciens* is shown (circle) when 132 (*Pseudomonas mosselii*) is in the middle of the NB plate.

Auxins such as IAA are an important class of plant growth hormones (phytohormones) (Gusmiaty et al., 2019), and auxin producing PSB might be PGPB by stimulating root growth and differentiation. The 25 PSB isolates were tested for IAA production, which was previously shown to act as a plant growth promoting phytohormone when produced by plant-associated bacteria (Lu et al., 2018; Lau et al., 2020).

Our results specifically showed that n00132 (*P. mosselii*) was the bacterium with higher (not significant) phosphate solubilizing index, (2.36 cm; Figure 2.2A) and significantly highest IAA production (10.81 µg mL^{-1}; Figure 2.2B). In addition, this bacterium was the only one with positive results for all indirect PGPB pathways including siderophore, cellulase, lipase, protease, and ammonia production.

Among all five bacterial species, *Brevibacillus* sp. was the only one that did not show positive effects on growth and development of the four rice varieties tested here, despite the fact that this bacterium is considered a PGPB with the positive *in vitro* PGPB assays of this study. According to RAST genomic analysis, *Brevibacillus* sp. isolated from *indica* Carolina 164 variety does not contain sequences for denitrifying reductase gene clusters, and this might be one of the reasons why this bacterium had no effect on growth and development of the four rice varieties tested here. Without denitrifying reductase gene clusters root formation and development in plants will not be efficient (Shen et al., 2013).

Interestingly, the results of this study showed that four sequenced bacteria were able to increase root and/or shoot growth and root or shoot biomass of two-week-old *japonica* Krasnodarskij 3352, *japonica* Zhonghua 11, and *indica* Kasalath varietal plants but had no (all five bacteria) or a negative (*P. mosselii* = n00132) effect on the growth of two-week-old *indica* Carolino 164 varietal plants tested here. Furthermore, it is interesting to note that *P. mosselii* isolated from leaf of the *indica* Carolino 164 variety had a positive shoot growth promoting effect on the *japonica* Krasnodarskij 3352 varietal plants but a negative effect on root growth of the rice variety it was isolated from (*indica* Carolina 164). More interestingly, the other bacterium (*P. rigui* = n00163) isolated from the root of *indica* Carolino 164 variety had a negative effect on root growth of the *japonica* Zhonghua 11 variety, but a positive effect on shoot growth of the *indica* Kasalath variety. The bacterium *Microvirga* sp. (n00170) was isolated from the leaf of Krasnodarskij 3352 and improved shoot growth of the *indica* Kasalath rice variety. Both *Microvirga* sp. and *P. graminis* (n00172) isolated from the leaf of Krasnodarskij 3352 improved root growth of the rice variety they were isolated from. For nutrient uptake ability, *P. mosselii* had a positive effect on the *japonica* Zhonghua 11 variety, while *Microvirga* sp. and *P. graminis* had negative effects on the *indica* Carolina 164 variety. These results reveal a distinct bacterial behavior when applied to different rice varieties. That is, some bacterial species were compatible with the rice variety they were isolated from and improved growth

and development of the local rice variety, while other bacterial species improved growth and development of non-local rice varieties.

To the best of our knowledge, this is the first work describing a positive plant growth promoting effect of phosphate solubilizing bacteria residing in two distantly related rice accessions on only one of the varieties they were isolated from as well as other rice varieties. There are at least two possibilities for this observation. First, genetic differences between the two varieties of *japonica* and the two varieties of *indica* plants assayed might lead to differential responses to bacterial metabolism (Vitte et al., 2004). It is reasonable to assume that PSB interact with and possibly regulate plant genes for growth and development. These genes could be differentially expressed in the four rice varieties or absent in one or two of the varieties. Second, there might be differences in how genes/operons and/or metabolic pathways within the five PSB respond to different exudates and plant hormones. Further studies need be done to understand the mechanisms of how these bacteria respond differently to different rice varieties, and *vice versa*, how different rice varieties respond differently to these bacteria. Additionally, we are interested in understanding the longer-term effects of PSB on plants, as the present work focused on the critical period of growth in the first two weeks. While many studies have discovered the benefits of plant growth promoting bacteria (Vitte et al., 2004; Raweekul et al., 2016; Yulianti & Rakhmawati, 2017; Yadav et al., 2017; Sharma et al., 2018), we demonstrate here that the benefits may not be universally applied. Even plant taxa as closely related as subspecies will respond differently to plant growth promoting bacteria, indicating the need to develop strain specificities between plants and bacteria.

2.5 Acknowledgements

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Chapter 3: *Pseudomonas mosselii* improves cold tolerance of Asian rice (*Oryza sativa* L.) in a genotype-dependent manner by increasing proline in *japonica* and reduced glutathione in *indica* varieties

Abstract

Cold stress is an important factor limiting rice production and distribution. Identifying factors that contribute to cold tolerance in rice is of primary importance. While some plant specific genetic factors involved in cold tolerance have been identified, the role of the rice microbiome remains unexplored. In this study, we evaluated the influence of plant growth promoting bacteria (PGPB) with the ability of phosphate solubilization on rice cold tolerance and survival. To reach this goal, inoculated and uninoculated two-week old seedlings were cold stressed and evaluated for survival and other phenotypes such as electrolyte leakage (EL) and necessary elements for cold tolerance. The results of this study showed that of the five bacteria, *Pseudomonas mosselii*, improved both *indica* and *japonica* varietal plants' survival and decreased EL, indicating increased membrane integrity. We observed different possible cold tolerance mechanisms in *japonica* and *indica* plants such as increases in proline and reduced glutathione levels, respectively. This bacterium also improved the shoot growth of cold exposed *indica* plants during the recovery period. This study confirmed the host genotype dependent activity of *P. mosselii* and indicated that there is an interaction between specific plant genes and bacterial genes that causes different plant responses to cold stress.

Keywords: *Oryza sativa* L., Cold tolerance, Plant growth promoting bacteria *Pseudomonas mosselii*, Proline, Reduced glutathione

3.1 Introduction

Global climate change causes major impacts on agriculture (Mendelsohn & Nordhaus, 2019). This has led many scientists to be concerned about climate change related to both increases of atmospheric greenhouse gas emissions and temperature of the Earth known as global warming (Alpana et al., 2017b). Global warming increases the occurrence of cold and warm extremes, known as the hiatus period (Johnson et al., 2018b). The occurrence of cold and warm extreme temperatures influences crop productivity.

Long winters significantly affect crop productivity because plants do not grow well in cold temperatures. Plants have developed different response mechanisms against cold environmental conditions that lead to improvement of their growth and development (Pessarakli, 2011). Phytohormones regulate growth and development of plants as well as defense pathways against cold environmental conditions (Pessarakli, 2011). In addition to innate plant defense mechanisms, different bacterial communities have particular importance in enhancing the cold tolerance of plants (Yadav et al., 2015). Several studies have shown that interactions between microbes and plants might cause plants to tolerate cold temperatures (Tibbett and Cairney 2007; Tiryaki et al. 2019; Acuna-Rodrıguez et al. 2020). The plant microbiome in cold environments has also developed physiological adaptations (Yadav et al., 2015). Cold-adapted bacteria are

known as psychrophilic microbes and primarily are members of phyla *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* (Yadav et al. 2015; Yadav et al. 2019). These bacteria also have the plant growth promoting abilities including phosphorus and zinc solubilization, nitrogen fixation, siderophore and plant hormone production that facilitate nutrient uptake and cold tolerance in plants (Verma et al., 2015). However, the functions of these plant-associated microbes during cold stress have not been specifically determined (Acuna-Rodrıguez et al., 2020).

Rice is one of the most important crops worldwide and a staple food for nearly three billion people (Mccouch et al., 2016). This plant is grown mostly in soils under wet conditions, known as flooded paddy fields (Lee et al., 2015). Asian rice plants (*Oryza sativa* L.) are usually grown in tropical and subtropical areas under mostly warm weather conditions (Xie et al., 2022). *O. sativa* has two major subspecies, *japonica* and *indica*. Human artificial selection allowed the relatively cold tolerant *japonica* subspecies be cultivated in temperate climates while the cold sensitive *indica* subspecies is mostly grown in warmer climates (Schläppi et al. 2017; Shakiba et al. 2017). While genetic determinants for this difference in cold tolerance have been studied (Schläppi et al. 2017; Shimoyama et al. 2020; Masclaux-Daubresse et al. 2010), very little is known about how the microbiome of rice might contribute to rice cold tolerance.

In this study, we focused on psychrotolerant and psychrotrophic bacteria with plant growth promoting abilities that inhabit the root endosphere and leaf tissues (known as endophytes) of both rice subspecies. Bacterial endosymbionts have highly beneficial effects on plants because of plant selection that invites specific bacterial species to enter their tissues (Saikkonen et al. 1998; Porras-alfaro and Bayman 2011; Wani et al. 2015; Rho et al. 2018). Different studies have looked at plant endophytes when plants were under cold stress conditions (Ruotsalainen and Kytöviita 2004; Barka et al. 2006; Upson et al. 2009; Theocharis et al. 2012; Murphy et al. 2014; Bylin 2014; Casler and Santen 2014; Abdel Hamed Latef and Chaoxing 2014; Zhou et al. 2015; Subramanian et al. 2015; Chen et al. 2016; Ghorbanpour et al. 2018; Xie et al. 2022). These studies have mostly focused on fungal endophytes and the microbial communities that benefit different plants under cold stress conditions. There is a lack of studies on the mechanism(s) of specific rice growth-promoting bacteria isolated from their host plants under cold stress. Moreover, to the best of our knowledge, none of these studies have investigated the effect of local bacterial communities on improving the cold stress tolerance potential of cold tolerant and cold sensitive rice subspecies. That is, the

response of one specific plant growth promoting bacterium to cold tolerance of two genotypically different rice subspecies is unknown. Previously (Maghboli Balasjin et al., 2022), we demonstrated that plant growth promoting bacteria isolated from one variety of rice do not necessarily stimulate growth of their own host under normal growth conditions and plant-microbe interactions are dependent on plant genotype and specific bacterial genes. These results were also supported by (Shalev et al., 2022) who showed that plant-microbe interactions are dependent on genetic differences in both plants and microbial species.

Therefore, based on our previous results, we hypothesized that specific psychrotrophic rice growth promoting endophytes contribute to the success of cold tolerance of different rice varieties through different mechanisms. To address our hypothesis, we selected five promising plant growth promoting bacteria (PGPB) with phosphate solubilizing ability, including *Pseudomonas mosselii* (n00132), *Paenibacillus rigui* (n00163), *Brevibacillus* sp. (n00167), *Microvirga* sp. (n00170) and *Paenibacillus graminis* (n00172), to inoculate seeds of both *indica* and *japonica* rice subspecies and evaluate their influence on rice cold tolerance. We chose phosphate solubilizing bacteria (PSB) because phosphorus is the second most important nutrient, after nitrogen, for plants in soil (Yulianti and Rakhmawati 2017). Phosphorus exists as insoluble phosphates in soil (Yulianti and Rakhmawati 2017). For plant roots to take up phosphate, insoluble inorganic phosphate needs to be converted to soluble phosphate (Chaiharn & Lumyong, 2009; Yulianti & Rakhmawati, 2017). Low temperature leads to inhibition of sucrose synthesis and consequently phosphate-limitation of photosynthesis, because sucrose is the end product of the photosynthesis process (Hurry et al., 2000). One way to help plants to tolerate low temperatures is to feed them with inorganic phosphate because it reverses the sucrose synthesis inhibition and therefore increases photosynthesis efficiency (Hurry et al., 2000). There are PSB that have the ability to solubilize inorganic phosphate in the soil and facilitate phosphate uptake for plants (Chaiharn & Lumyong, 2009; Zhang et al., 2011; Yulianti & Rakhmawati, 2017). Therefore, it is important to isolate PSB and evaluate their influence on plants before and after cold stress. The main goal of this study was to identify the mechanism of actions of PGPB in their own host and other genotypically different rice varieties, when rice plants were under cold stress. This research determined whether PGPB can help agricultural productivity in cold environments by mediating different processes such as maintenance of nutrients and fighting against cold stress.

3.2 Materials and methods

3.2.1 Bacterial isolation from roots and leaves of *Oryza sativa*

Bacteria used in this study were isolated from plants grown in our laboratory and their isolation process and details about the soil mixture and its nutrient contents have been fully described in a previous publication (Maghboli Balasjin et al., 2022; see chapter 2). Two subspecies of *O. sativa* including Krasnodarskij 3352 [Genetic Stock Oryza (GSOR) no. 311787], representing *japonica*, and Carolino 164 (GSOR no. 311654), representing *indica*, were used for this study. Based on the results, 5 PGPB performing a variety of activities were genotyped through whole genome sequencing (Maghboli Balasjin et al., 2022). All information about sequence reads can be found in a previous publication (Maghboli Balasjin et al., 2022). The sequences were deposited at the NCBI Sequence Read Archive (SRA) under BioProject no. PRJNA667792 and SRA accession no. SRS7484588 to SRS7484592.

3.2.2 Bacterial phenotypic characterization for plant growth promoting activities at low temperatures (4˚C and 10˚C)

Psychrotrophic abilities of the five PGPB were determined at 4˚C and 10˚C (the same temperatures that were applied to rice plants). Growth at 30˚C was tested as a control temperature. For 10˚C and 30˚C, two separate 96 well microplates were used. 200 µL of Lysogeny broth (LB) medium was added to each well of the microplate and one colony from each of 5 the five PGPB was mixed with LB. For each bacterium, 3 wells were inoculated to have 3 replicates. Microplates were incubated at 10˚C and 30˚C in SpectraMax plus 384 for 48 h. The hours of operation for Optical Density (OD600) were 0, 2, 4, 6, 16, 19, 21, 23 and 48. For 4˚C, 5 tubes containing 5 mL LB were inoculated with one colony from each of the five PGPB and incubated at 4˚C for 96 h. This experiment was done two times independently. The hours of operation for Optical Density (OD600) were 48 and 96.

All five isolated PGPB were characterized for plant growth promoting activities through culture-based and colorimetric assays at the temperatures that were applied for plant cold stress (4˚C and 10˚C). Assays included indoleacetic acid (IAA) production, phosphate solubilization, nitrogen fixation, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, lipase production, casein hydrolyzing, siderophore and cellulase production and an *in vitro* assay for salt tolerance as described previously

(Maghboli Balasjin et al. 2022). Plates were incubated at 4˚C and 10˚C for 14 d, then 4˚C plates were incubated at 28˚C for one week.

3.2.3 Seed inoculation with bacteria and plant growth

To avoid growth of other soil microorganisms, all young rice seedlings were grown hydroponically instead of in soil. Seeds of both subspecies were inoculated with a bacterial suspension containing 10^8 colony forming units (CFU) following our previous method (Maghboli Balasjin et al., 2022). Seeds were dehulled and surface sterilized by immersing them first, in 70% (v/v) EtOH (1 min, then 3x rinsed in sterile water), and second, in 70% (v/v) sodium hypochlorite (5 min, then 3x rinsed in sterile water). Surface sterilized seeds were soaked in a 10^8 CFU bacterial suspension in 0.85% (w/v) KCl and placed on a shaker overnight (200 rpm) at room temperature (22˚C – 23˚C) [control plants: seeds inoculated in 0.85% (w/v) KCl]. Soaked seeds were germinated on solid Murashige-Skoog (MS) medium at 30˚C for 48 h, then transferred into wells of qPCR strips with the bottom tip cut away and placed into magenta boxes containing autoclaved distilled deionized water (ddH_2O) and placed in a growth chamber with cycles of 12 h of light (28˚C) and 12 h of dark (24˚C). Every other day the magenta boxes were filled with sterile ddH_2O. After 10 d of growth, ddH_2O in each box was replaced with a liquid full-strength MS medium diluted 4x with ddH_2O (1/4 strength MS) as fertilizer. Plants remained in the growth chamber for 14 d. A total of 180 *japonica* and 180 *indica* plants were grown for each trial. Half of the *japonica* and *indica* seeds were inoculated with PGPB (90 inoculated and 90 uninoculated *japonica* and *indica* seeds, each). Inoculated and uninoculated seedlings were placed in 6 magenta boxes (30 inoculated and 30 uninoculated plants per magenta box) as biological replicates. A total of 6 magenta boxes were evaluated for cold tolerance experiments in each trial (3 independent trials in total). There was a one-week interval between each trial of bacterial-seed inoculation experiment.

3.2.4 Cold stress

Based on studies by Schläppi et al. (2017) for low temperature seedling survivability (LTSS) measurements, cold stress was applied to 2-week-old rice seedlings, which had reached the 2-leaf stage of development. To coordinate microbiome experiments with LTSS measurements, we thus used 2-week-old seedlings were used as an early vegetative stage providing enough root biomass for various assays. To directly compare LTSS experiments for *indica* and *japonica* plants, we used temperature treatments that

lead to half plant population survival (LT50=50% LTSS) were used, as previously determined (Shi et al., 2020), with the following modifications: duration of cold stress was the same (4 d) for both subspecies, with the cold temperature regime at 4˚C for the relatively cold tolerant *japonica* plants and 10˚C for the cold sensitive *indica* plants. Two-week-old inoculated and uninoculated *japonica* and *indica* plants were transferred for 4 d to 4˚C and 10˚C chambers, respectively, with 12 h/12 h light/dark cycles (cold stress treatment) and put back for 1 week to a growth chamber with 12 h light/28˚C and 12 h dark/24˚C cycles (recovery growth period). At each time point, plant samples were collected for further analysis.

To avoid temperature fluctuations during the cold stress period, growth chamber doors remained closed and no irrigation occurred. During the recovery growth period, irrigation occurred every other day.

3.2.5 Electrolyte leakage (EL)

Electrolyte leakage (EL) measurements were performed following the methods of Shi et al. (2020) using the LAQUAtwin B-771 conductivity meter (Horiba Scientific, Japan). To ensure that there were no free ions in the ddH_2O that would influence conductivity, only glass tubes predetermined to have 1 micro Siemens (1 µS) or less of conductivity before transferring leaves were used. The percentage (%) of EL was calculated by dividing the initial EL by the total EL x 100. From each replicate, five plants were randomly chosen. A total of 15 EL measurements from inoculated and uninoculated plants were done for each trial (5 inoculated and 5 uninoculated plants were randomly selected from each of the 6 magenta boxes). This experiment was repeated three times, independently.

3.2.6 Low temperature seedling survivability (LTSS)

At the end of the day-7 recovery period following the 4 days of cold treatment, the number of living, green and healthy-looking plants was counted and the final percentage of LTSS was calculated as below:

%LTSS = Number of the surviving, green and healthy plants after one week of recovery / Total number of green and healthy plants before cold stress

3.2.7 Shoot and root growth measurements at 15˚C

To be consistent with LTSS and EL experiments, inoculated and uninoculated rice plants were grown in a growth chamber at 12 h light/12 h dark and 28˚C/25˚C cycles for 14 days after which plants were transferred to a 15˚C chamber at 12 h light/12 h dark cycles for 10 days. Ten days of incubation was

chosen to allow enough time for root and shoot growth at 15˚C. Shoot and root length measurements in cm were done at 5 time points: (1) when plants were 14 days old, (2) when plants were at 15˚C for 5 days, (3) when plants were at 15˚C for 10 days, (4) when plants were back in the growth chamber (recovery period) for 5 days, and (5) when plants were back in the growth chamber (recovery period) for 10 days. This experiment was repeated three times, independently. Each trial contained two replicates (two boxes containing similar rice plants variety with the same treatment).

For all following measurements described below, there was a one-week interval between each trial of a bacterial-seed inoculation experiment, and sample collection from inoculated and uninoculated plants was done at three time points: (1) before cold stress (two-week old plants), (2) immediately after cold stress, and (3) after 7 d of recovery growth.

3.2.8 Phosphate measurements

Collected tissues were ground in liquid nitrogen and weighed. The ascorbic acid assay method (Murphy & Riley, 1962) was used to measure soluble phosphate levels in plant tissues with minor modifications. Briefly, 1 mL sterile ddH_2O was added to each ground sample. Samples were vortexed for 2 min and centrifuged 10 min at 12,052 x g and 160 µL of the supernatant of each sample transferred to new 1.5 mL sterile tubes (the rest of the samples were saved for nitrate measurement, see below). A mixture of 5 N H_2SO_4 (50 mL) (mix 70 mL H_2SO_4 in 500 mL ddH_2O), potassium antimonyl tartrate (5 mL) (stock solution: 1.3715 g of potassium antimonyl tartrate in 500 mL ddH_2O), ammonium molybdate (15 mL) (stock solution: 20 g of ammonium molybdate in 500 mL ddH_2O), and ascorbic acid (30 mL) (stock solution: 0.88 g of ascorbic acid in 50 mL ddH_2O) was prepared, and 1 mL of the mixture added to 160 µL of the supernatant of each sample followed by incubation for 20 min at room temperature. The presence of potassium antimonyl tartrate and ammonium molybdate causes orthophosphate to create a blue color. Sulfuric acid converts polyphosphates to orthophosphate and forms an antimony-phospho-molybdate complex. Ascorbic acid causes the reduction of this complex to an intensely blue-colored complex. After 20 min of incubation at room temperature, total orthophosphate was measured by the direct colorimetric analysis procedure at 880 nm wavelength. A standard curve was created using a KH_2PO_4 (1 M) stock solution for calculating mM amounts of orthophosphate. Results were normalized by mg fresh tissue

weight. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

For determining the ability of PGPB to solubilize inorganic phosphate at low temperatures, cold tolerant *japonica* rice seeds were inoculated with bacterial suspensions and two types of MS media (chemical composition shown in Table 3.1) were used: (1) homemade MS medium -KH_2PO_4 +$Ca_3(PO_4)_2$ (missing soluble potassium phosphate and containing insoluble calcium phosphate) and (2) homemade MS -KH_2PO_4 (missing soluble potassium phosphate). *Japonica* seeds were surface sterilized as described before (Maghboli Balasjin et al., 2022) and half of them were soaked in bacterial suspension in 0.85% (w/v) KCl and the other half soaked in KCl alone as control. Inoculated and uninoculated seeds were germinated for 48 h at 30˚C on solid homemade MS medium -KH_2PO_4 +$Ca_3(PO_4)_2$ and MS medium -KH_2PO_4. Germinated seeds were transferred to magenta boxes containing ¼ strength liquid homemade MS media described above and placed in a growth chamber with cycles of 12 h of light (28˚C) and 12 h of dark (24˚C). Every other day the magenta boxes were filled with sterile ddH2O. On day 14, plants were transferred to a 4˚C chamber with 12 h light/12 h dark cycles for 4 days. At each time point (before and immediately after cold stress), plant samples were collected for further analysis. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

Table 3.1 Ingredients of the homemade MS medium to get 1x solution.

MS Macronutrients (10x)	**g/L**
KNO3	19
NH4NO3	16.5
CaCl2.2H2O	4.4
MgSO4.7H2O	3.7
KH2PO4 → if replaced with Ca3(PO4)2	1.7 → 3.87
MS Micronutrients (1000x)	**g/L**
MnSO4.4H2O	22.3
ZnSO4	8.6
H3BO3	6.2
KI	0.83
Na2MoO4.2H2O	0.25
CuSO4.5H2O	0.025
CoCl2.6H2O	0.025
FeSO4 EDTA Iron (100x)	**g/L**
FeSO4.7H2O	2.78
Na2EDTA	3.72

3.2.9 Nitrate (NO3) measurements

The same samples that were used for the phosphate measurements (the ones with only ¼ strength regular MS which was added on day 10) were also used for nitrate measurements using a LAQUAtwin B-743 (Horiba Scientific, Japan) nitrate meter. 120 µL of the supernatant of each sample was applied to the meter and the amount of NO_3 in ppm was measured. Calculations to obtain the amount of NO_3 in µg mg^{-1} of fresh tissue were done as below:

$$NO3\ (\mu g\ mg^{-1}) = \left[\frac{\{NO3(ppm)\times 10^{-6}\}\times 10^{2}}{tissue\ weight(g)\times 10^{3}}\right] \times 10^{3}$$

This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

3.2.10 Protein amounts

A colorimetric assay (Bradford method) for protein content quantification in plant tissues was used following the methods of Bonjoch and Tamayo (2001) using Coomassie® Brilliant Blue G-250 Bradford reagent with minor modifications. Collected tissues were ground in liquid nitrogen and weighed. Phosphate buffer (0.1 M, pH = 7; containing 0.0536 M K_2HPO_4 and 0.0464 M KH_2PO_4 in 500 mL ddH_2O) was prepared and 1 mL was added to each ground sample. Samples were incubated for 30 min at room temperature (22˚C - 23˚C). During incubation, samples were inverted gently every 5 min. Samples were then centrifuged at 13,148 x g for 10 min and 100 µL of each supernatant was transferred to a new 1.5 mL tube containing 1 mL of the Bradford reagent and mixed (protein-dye binding step). Samples were placed on a rotatory shaker (200 rpm) at room temperature (22˚C - 23˚C) for 20 min. A standard curve was created using the Bradford reagent and a 1 mg μL^{-1} Bovine Serum Albumin (BSA) stock solution. Absorbance of each sample was measured at 595 nm wavelength using a spectrophotometer. Results were normalized against mg fresh tissue weight. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

3.2.11 Chlorophyll content

The chlorophyll content of inoculated and uninoculated *japonica* and *indica* plants were measured following the methods of Ritchie (2006) with minor modifications. Briefly, fresh leaf tissues were soaked

in 6 mL 100% ethanol and incubated in the dark at room temperature for 4 d. Then, leaves were taken from the ethanol and incubated at 50˚C for 24 h to obtain the dry weight of each leaf tissue. The absorbance of the tubes containing ethanol was measured at 649 and 665 nm wavelengths using a spectrophotometer for chlorophyll *b* and *a*, respectively. Pure ethanol was used as blank. The chlorophyll contents of each plant were calculated as follows:

$$\text{Chlorophyll } a: 13.70 \times A665 - 5.76 \times A649\ \mu g\ mL^{-1}$$

$$Chlorophyll\ b: -7.60 \times A665 + 25.8 \times A649\ \mu g\ mL^{-1}$$

All numbers were multiplied by 6, because leaf tissues were initially soaked in 6 mL 100% ethanol. Amounts of chlorophyll *a* and *b* were normalized by mg of dry weight of leaf tissues. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

3.2.12 Sugar amounts

The sugar content in plant tissues was measured using the two steps of sugar extraction and colorimetric sugar staining following the methods of Leach and Braun (2016) and Kerepesi et al. (1996), respectively, with minor modifications. Collected tissues were ground in liquid nitrogen, weighed, and 1 mL of 12:5:3 methanol;chloroform:water (MCW) extraction buffer containing lactose (0.1 µg μL^{-1}) was added to each sample on ice. Samples were vortexed briefly and incubated in a 50˚C water bath for 30 min. After incubation, samples were centrifuged at room temperature (12,052 x g, 5 min). The supernatant from each tube was transferred to new 15 mL conical tube and placed on ice. 1 mL of MCW extraction buffer without lactose was added to plant tissues. The tubes were centrifuged at room temperature (13,148 x g, 5 min) and the supernatant from each sample was transferred to the new 15 mL conical tube. This step was repeated one more time and, each time, a pipette tip was used to further break down plant tissues. Conical tubes were kept on ice during the time. As a final step in sugar extraction, sterile ddH_2O (0.6 volume of the total collected supernatant amount in each tube) was added to the tubes. Samples were vortexed for 10 s and centrifuged at room temperature (4 650 x g, 5 min). The amount of 1.5 mL of the aqueous top solution (sugar extract) in each conical tube was transferred to a 1.5 mL microcentrifuge tube and stored at -20˚C overnight. For the colorimetric assay, 40 µL of each sample was mixed with 200 µL of 5% phenol and 1

mL of concentrated sulfuric acid. Samples were incubated at room temperature (20 min). Finally, the absorbance of samples was measured at 510 nm. A standard curve was created using a 1 mg mL^{-1} sucrose stock solution. Results were normalized by mg fresh tissue weight. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

3.2.13 Proline amounts

Proline content was measured with a colorimetric assay following the protocol of Carillo & Gibon (2016) with minor modifications. Briefly, two-week old inoculated and uninoculated (control) whole plant tissues were collected for measuring the proline content. Collected tissues were ground in liquid nitrogen and weighed. 1 mL 70% ethanol was added to each sample and vortexed briefly and centrifuged (13,148 x g, 5 min). 500 µL of the supernatant of each sample was transferred to another 1.5 mL tube and 1 mL of reaction mix [mixed ninhydrin (1% w/v) with glacial acetic acid (60% v/v) and ethanol (20% v/v)] was added. Tubes were inverted gently and incubated in a 95˚C heat block for 20 min. After incubation, tubes were centrifuged (6 149 x g, 1 min) and cooled down. The absorbance of samples was measured at 520 nm. A standard curve was created using a 1 M proline stock solution. Results were normalized to mg fresh tissue weight. This experiment was repeated independently two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

3.2.14 Reduced glutathione amounts

Reduced glutathione measurements in plant tissues were done using the colorimetric assay of Sahoo et al. (2017) with minor modifications. Chemical composition of each solution used is shown in Table 3.2. Collected whole plant tissues were ground in liquid nitrogen and weighed. The amount of 1 mL of 6% metaphosphoric acid with 1mM EDTA was added to each sample and centrifuged at 4˚C (13,148 x g,15 min). 400 µL of supernatant of each sample was transferred to a new tube containing 1 mL 0.5 M potassium phosphate buffer. 100 µL of 2-nitrobenzoic acid (DTNB), 200 µL of bovine serum albumin (BSA) and 100 µL of nicotinamide adenine dinucleotide hydrogen (NADH) were added to each sample. Tubes were incubated in a 37˚C water bath for 15 min in the dark. After cooling down, the absorbance of samples was measured at 412 nm. A standard curve was created using a 1 M reduced glutathione stock solution. Results were normalized by mg fresh tissue weight. This experiment was repeated independently

two more times with two separate inoculated and uninoculated plant growing trials. For each trial, three plant samples were collected.

Table 3.2 solutions for reduced glutathione measurement.

Solutions	**Recipe**
6% metaphosphoric acid containing 1 mM EDTA	6 g metaphosphoric acid and 29.224 mg EDTA in 100 mL ddH_2O
Potassium phosphate buffer (0.5 M, pH = 7.5)	8.709 g K_2HPO_4 and 6.8045 g KH_2PO_4 in 100 mL ddH_2O
DTNB (10 mM) (light sensitive)	99 mg DNTB in 25 mL ethanol (store at -20˚C)
BSA (10 mM)	Dissolve 6.6 g BSA in 10 mL ddH_2O (store at -20˚C)
NADH (0.5 mM)	8.292 mg in 25 mL ddH_2O

3.2.15 Nitro Blue Tetrazolium (NBT) Staining

The nitro blue tetrazolium (NBT) indicator was used to evaluate levels of reactive oxygen species (ROS) in rice leaf tissues, which is specified for visualization of $O^{-}{}_2$ in plant leaves, following methods described in Shi et al. (2020) with minor modifications. Briefly, the light sensitive 0.02% NBT solution was made by adding 0.1 g of 0.02% NBT powder into 5 mM phosphate buffer containing 5.054 g $Na_2HPO_4.7H_2O$, and 0.848 g $NaH_2PO_4.H_2O$ in 500 mL deionized H_2O (pH = 7.5). The second leaf of each plant sample was collected from two-week old inoculated and uninoculated (control) plants for NBT staining at three time points: (1) before cold stress (two-week old plants), (2) after cold stress, and (3) 3 days into the recovery period. Collected leaf tissues were placed in tubes containing 0.02% NBT solution and incubated in the dark at room temperature overnight. The next day, the NBT solution was taken out and replaced with 100% ethanol. Tubes were incubated in the dark at room temperature for 3-4 h, then placed in boiling water until the ethanol was evaporated. Leaf tissues were taken out and placed on a white background for picture taking. This experiment was done three times independently. Each trial contained 3 leaves of three plants for each time point. Pictures were analyzed using Image-J software.

3.2.16 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Expression levels of proline and reduced glutathione synthesis genes in inoculated and uninoculated rice plant tissues were measured using the quantitative polymerase chain reaction (qPCR) method. Specific primers for proline and reduced glutathione genes were designed using the https://www.idtdna.com/pages/products/genes-and-gene-fragments site. Primer sequences are shown in Table 3.3.

Table 3.3 The Oligo-dT primer for making cDNA and primer sequences designed for measuring proline and reduced glutathione transcripts in *japonica* and *indica*, respectively.

Primer	**Sequence**
OsPRODH Forward	AGCAGATCATCCCGTACCT
OsPRODH Reverse	ACTCACGTCCCAGCATTG
OsP5CR Forward	GGGATCTTGCACTTGGTCTT
OsP5CR Reverse	CCCTTCTCAAGCTCTTGTATCC
OsGR3 Forward	GAGACTCCTCAAGCAGTAA
OsGR3 Reverse	CCCACAGCCCATATAGAA
OsACT Forward	TCCTGATGGACAGGTTATC
OsACT Reverse	CTTCATGATGGAGTTGTATGT
Oligo-dT	GGCCACGCGTCGACTACTTTTTTTTTTTTTTT

Total RNA was isolated from *japonica* and *indica* tissues using the phenol: chloroform method as described by Chan et al. (2007), with minor modifications, or by using the TRIzol reagent (ThermoFisher). After finishing the procedures for RNA isolation, 30 µL and 20 µL of diethyl pyrocarbonate (DEPC)-treated ddH_2O was added to the dried RNA pellet from *japonica* and *indica*, respectively, and tubes were incubated at 65˚C in a water bath for 30 min to dissolve the pellet.

Before reverse transcription, DNA contamination was removed from total RNA samples using a DNase kit according to manufacturer's instructions (Invitrogen, DNase - Micro Kit). The supernatant containing total RNA was transferred to a fresh 1.5 tube and placed on ice before making complementary DNA (cDNA) using the SuperScript IV Reverse Transcriptase (RT) kit according to manufacturer's instructions (Invitrogen). First-strand cDNA samples were stored at -80˚C prior to qPCR analysis.

For qPCR analysis, 1 µl of 10-fold diluted first-strand cDNA, 0.5 µl of forward and reverse primers (both 12 mM), 10 µl of 2x universal SybrGreen supermix (BioRad), and 8 µl of DEPC H_2O were mixed and added to PCR wells and reactions run under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min using a CFX96 Touch™ Real-Time PCR Detection system (BioRad). The $2^{-\Delta\Delta CT}$ method was used to quantify qPCR reactions using *OsACTIN* as internal control. Reaction products for which melt curves showed a primer dimer peak were excluded from the quantifications.

3.2.17 Statistical analysis

All statistical comparisons (two-tailed student *t*-test & one-way ANOVA with post-hoc Tukey HSD Test) were done using Minitab statistical software v20.2.0.0 (free access from University of Wisconsin Milwaukee).

3.3 Results

3.3.1 *P. mosselii* improves cold stress potential of inoculated rice plants

Results from growth at 10˚C showed that *Pseudomonas mosselii* (n00132) was psychrotolerant, that is, able to grow at temperatures below 20˚C but optimally at 30˚C, while the other four PGPB, *Paenibacillus rigui* (n00163), *Brevibacillus* sp. (n00167), *Microvirga* sp. (n00170), and *Paenibacillus graminis* (n00172), showed better growth at 4˚C (Figure 3.1), indicating that they were psychrophilic, that is, able to grow below 5˚C but optimally at 15˚C. Results from plant growth promoting assays showed that all five PGPB were positive for different plant growth promoting assays at 10˚C, while at 4˚C, they were only slight positive for these assays. When 4˚C plates were incubated at 30˚C for one week, the results were similar to those at 10˚C (Table 3.4, Figure 3.2). Interestingly, psychrotolerant *P. mosselii* had a significantly higher phosphate solubilizing ability at 4˚C than *Paenibacillus rigui* and *Microvirga* sp. (Figure 3.2).

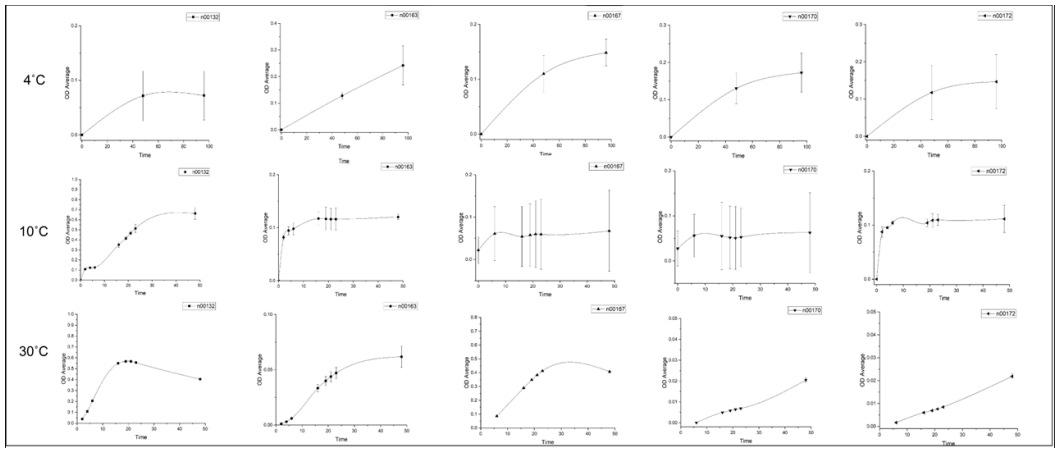

Figure 3.1 Growth curves of 5 isolated PSB at low temperatures 4˚C and 10˚C. Each of these 5 isolates were named based on the codes that were initially given. n00132 = *Pseudomonas mosselii*, n00163 = *Paenibacillus rigui*, n00167 = *Brevibacilllus* sp., n00170 = *Microvirga* sp., n00172 = *Paenibacillus graminis*.

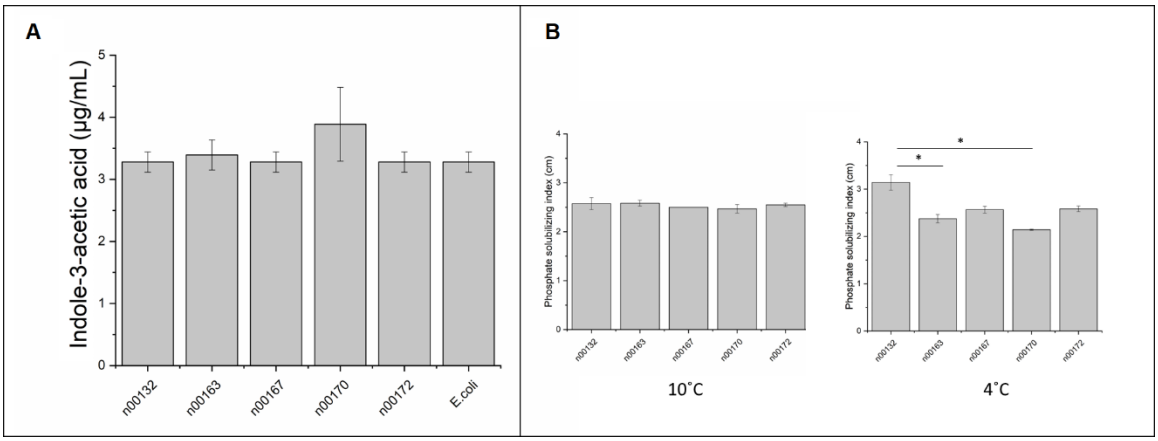

Figure 3.2. Indole acetic acid (IAA) production at 4˚C and phosphate solubilization by 5 isolated PSB at 4˚C and 10 ˚C. **A.** There was no difference between the 5 PSB in IAA production at 4˚C. *E. coli* strain OP50 was used as the positive control for IAA production. No significant difference was observed compared to *E. coli*. Statistical analyses: two-tailed student *t*-test. **B.** Phosphate solubilization index at 4˚C and 10˚C. No significant difference was observed compared each of these PSB at 10˚C. For the set of the plates which were incubated at 4˚C for two weeks and incubated at 28˚C for one more week, phosphate solubilization of n00132 isolate increased significantly compared to n00163 and n00170. Statistical analyses: one-way ANOVA and post-hoc Tukey HSD Test. *: $p < 0.05$
Each of these 5 isolates were named based on the codes that were initially given. n00132 = *Pseudomonas mosselii*, n00163 = *Paenibacillus rigui*, n00167 = *Brevibacilllus* sp., n00170 = *Microvirga* sp., n00172 = *Paenibacillus graminis*.

Table 3.4 Plant growth promoting (PGP) culture-based assay results for 5 isolated PSB. All plates were incubated at 4˚C and 10˚C for 14 d. Plates which were incubated at 4˚C for 14 d, transferred to 30˚C for 1 week. For nitrogen fixation, nitrogen-rich yeast extract-mannitol agar containing Congo red dye (CR-YMA) was used. Colors of the bacterial colonies were categorized as ++ (Pale white-pink), + (White-pink), - (Strong pink-red). The less red the colonies, the more nitrogen they can fix.

PGP assays	*Pseudomonas mosselii*	*Paenibacillus rigui*	*Brevibacilllus* sp.	*Microvirga* sp.	*Paenibacillus graminis*
CR-YMA	4˚C: ++ 10˚C: ++	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: ++ 10˚C: ++	4˚C: + 10˚C: +
Lipase	4˚C: + 10˚C: +	4˚C: - 10˚C: -	4˚C: - 10˚C: -	4˚C: - 10˚C: -	4˚C: - 10˚C: -
Siderophore	4˚C: + 10˚C: +	4˚C: - 10˚C: -	4˚C: - 10˚C: -	4˚C: + 10˚C: +	4˚C: - 10˚C: -
Casein hydrolyzing	4˚C: + 10˚C: +	4˚C: - 10˚C: -	4˚C: - 10˚C: -	4˚C: - 10˚C: -	4˚C: - 10˚C: -
ACC deaminase	4˚C: Strong + 10˚C: Slight +	4˚C: Strong + 10˚C: Slight +	4˚C: Strong + 10˚C: Slight +	4˚C: Strong + 10˚C: Slight +	4˚C: Strong + 10˚C: Slight +
LB + 2% NaCl	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +
LB + 4% NaCl	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +
LB + 6% NaCl	4˚C: Slight + 10˚C: -	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +	4˚C: + 10˚C: +
LB + 8% NaCl	4˚C: - 10˚C: -	4˚C: Slight + 10˚C: Slight +	4˚C: Slight + 10˚C: Slight +	4˚C: Slight + 10˚C: Slight +	4˚C: Slight + 10˚C: Slight +

To investigate the influence of the five previously isolated PGPB (Maghboli Balasjin et al., 2022) *Pseudomonas mosselii* (n00132), *Paenibacillus rigui* (n00163), *Brevibacillus* sp. (n00167), *Microvirga* sp. (n00170), and *Paenibacillus graminis* (n00172) on the cold tolerance potential of rice seedlings, a total of 90 two-week old plants for each of the cold tolerant *japonica* and cold sensitive *indica* varietal groups (both inoculated and uninoculated control plants) were cold treated at 4˚C (*japonica*) and 10˚C (*indica*) for 4 days. Immediately after the cold treatment, 4 pieces of the middle part of the second leaf of each of 5 rice plants per replicate were removed and electrolyte leakage (EL) from leaf cells into deionized water determined. This EL measurement determined how much damage leaf cell membranes sustained during the cold treatment (the higher EL, the more damage). This showed that both *japonica* and *indica* plants inoculated with *P. mosselii* had significantly lower ($p < 0.05$) EL (16% and 14%, respectively) than uninoculated rice plants (20% and 18%, respectively) (Figure 3.3). While only *P. mosselii* had a positive effect on *japonica* plants (Figure 3.3A), compared to uninoculated plants, *P. rigui* also helped significantly lower EL in *indica* plants (Figure 3.3B).

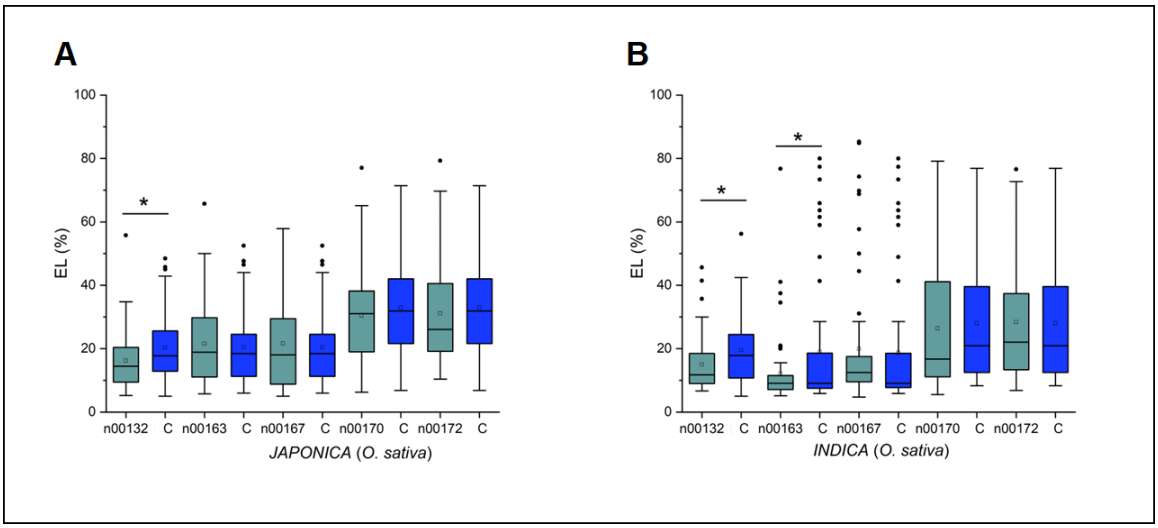

Figure 3.3 Percent (%) Electrolyte Leakage (EL) in leaves of rice plants inoculated (green) with five phosphate solubilizing bacteria (PSB) compared to uninoculated (blue) plants after cold stress. **A.** *Japonica* plants inoculated with *P. mosselii* (n00132) had significantly lower EL compared to uninoculated (C) control plants. **B.** *Indica* plants inoculated with *P. mosselii* (n00132) and *P. rigui* (n00163) had significantly lower EL compared to uninoculated (C) control plants. ___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$

After the 4 days of cold exposure, plants were returned to regular warm growth temperatures for a recovery period of one week, after which the number of green and healthy-looking plants were counted and

compared to the total number of plants subjected to the cold stress treatment. Like the EL results, the results showed that only *P. mosselii* had a positive effect on the survival of both *japonica* (74% of inoculated compared to 34% of uninoculated) and *indica* (66% of inoculated compared to 55% of uninoculated) plants exposed to cold temperatures (Figures 3.4 and 3.5). Therefore, due to its demonstrated positive effect on improving cold tolerance potential of both *indica* and *japonica* plants, we focused on *P. mosselii* to determine its mechanism(s) of action inside plant tissues.

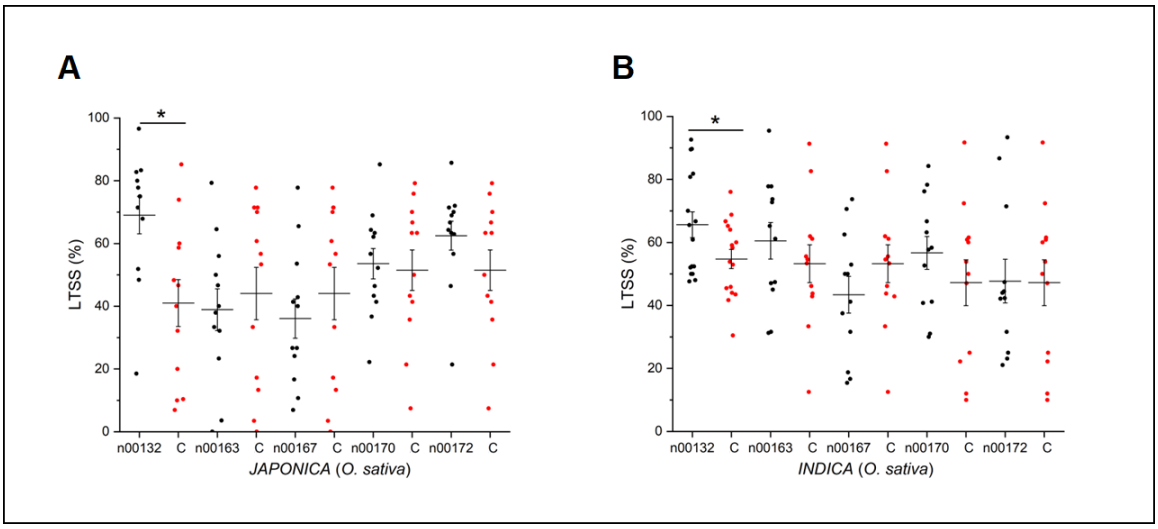

Figure 3.4 Percent (%) Low Temperature Seedling Survivability (LTSS) of rice plants inoculated (black dots) with five phosphate solubilizing (PSB) bacteria compared to uninoculated (red dots) plants after one week of recovery. **A.** *Japonica* plants inoculated with *P. mosselii* (n00132) had significantly higher LTSS compared to uninoculated (C) control plants. **B.** *Indica* plants inoculated with *P. mosselii* (n00132) had significantly higher LTSS compared to uninoculated (C) control plants. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$

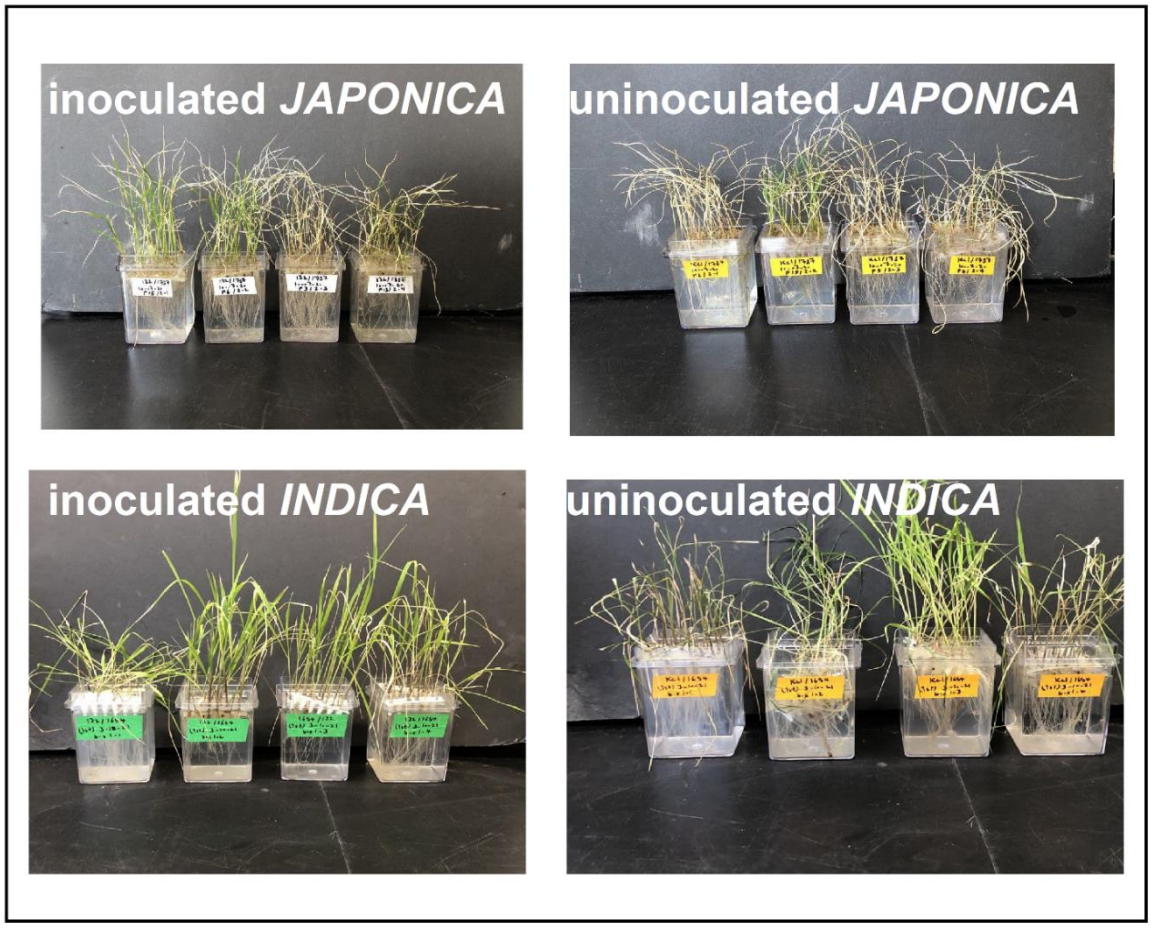

Figure 3.5 Inoculated *japonica* and *indica* plants with *Pseudomonas mosselii* had higher survival (LTSS) compared to their uninoculated control plants.

3.3.2 Effect of *P. mosselii* on shoot and root growth at 15˚C

The isolated *P. mosselii* is a psychrotrophic bacterium that can grow and survive at 4˚C and 10˚C (Table 3.4, Figure 3.1). To determine whether *P. mosselii* had a positive effect on growth and development of rice plants at the threshold temperature of 15˚C, root and shoot growth of *P. mosselii* inoculated and uninoculated *indica* and *japonica* rice plants was measured at 5 time points. Based on the results, *P. mosselii* inoculated *japonica* rice plants generally did not show improvement in shoot and root growth compared to uninoculated plants. However, *P. mosselii* inoculated *indica* plants had significantly improved ($p < 0.05$) shoot growth by 30% between days 5 and 10 of the recovery period compared to uninoculated control plants (Figure 3.6) and this was the only difference that was observed. In addition, when comparing shoot growth between day 10 of 15˚C cold and day 10 of the recovery period, for both inoculated and uninoculated *indica* plants, 12% growth for inoculated plants and 9% growth for control plants was

observed, respectively. Therefore, *P. mosselii*, originally isolated from leaves of *indica* plants (Maghboli Balasjin *et al.* 2022), had a positive effect on shoot growth of *indica* plants at the threshold temperature of 15˚C.

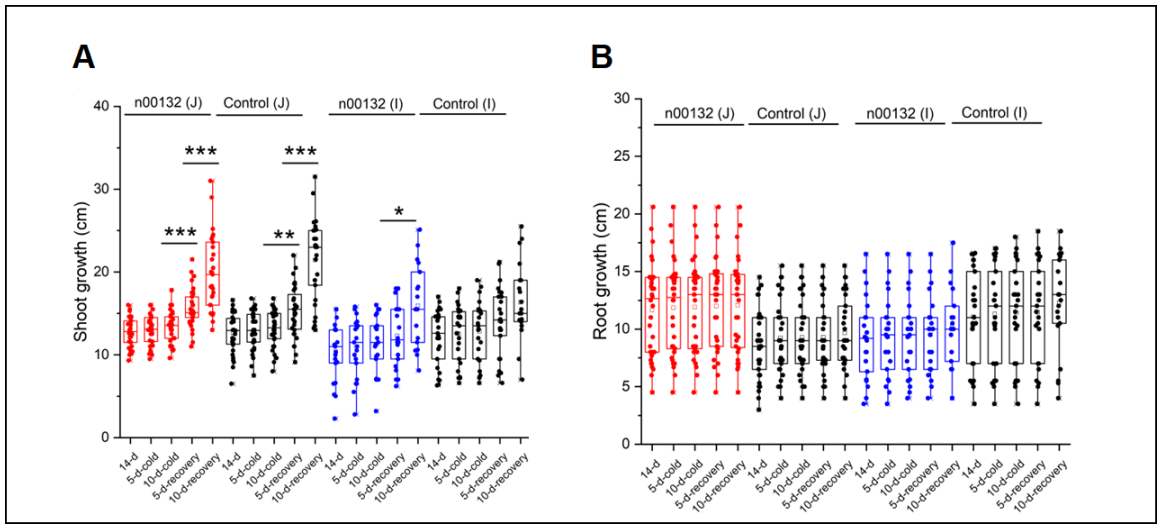

Figure 3.6 Shoot and root growth of inoculated (red and blue dots) and uninoculated (black dots) *japonica* (J) and *indica* (I) plants. **A.** *Japonica* plants inoculated with *P. mosselii* (n00132) and uninoculated *japonica* plants (control) showed significantly improved shoot growth from day 10 in 15˚C to day 10 of recovery, while only *indica* plants inoculated with *P. mosselii* (n00132) showed significantly improved shoot growth from day 5 to day 10 of recovery. **B.** No significantly improved root growth was observed in both *japonica* and *indica* plants. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

3.3.3 *P. mosselii* reduces nitrate levels in rice plant tissues

Previously, Maghboli Balasjin et al. (2022) showed that *P. mosselii* had the ability of phosphate solubilization and nitrogen fixation among other isolated rice-associated bacteria. In this study, the influence of this bacterium on the amounts of phosphate and nitrate inside rice tissues was tested. The results revealed that there was no increase in phosphate amounts in either *japonica* or *indica P. mosselii* inoculated plants compared to control (KCl) plants at the three time points (Figure 3.7A & B). However, the amount of nitrate in *japonica* control plants was 19% higher after the recovery period than in inoculated plants (Figure 3.6C), while in *indica* control plants, the amount of nitrate was 42% higher both before and after cold treatments in control than inoculated plants (Figure 3.7D).

Note: for phosphate measurement in this section, ¼ MS [commercial powder (ready to use)] was added to plants on day 10 and the amount of soluble phosphate in plant tissues was measured.

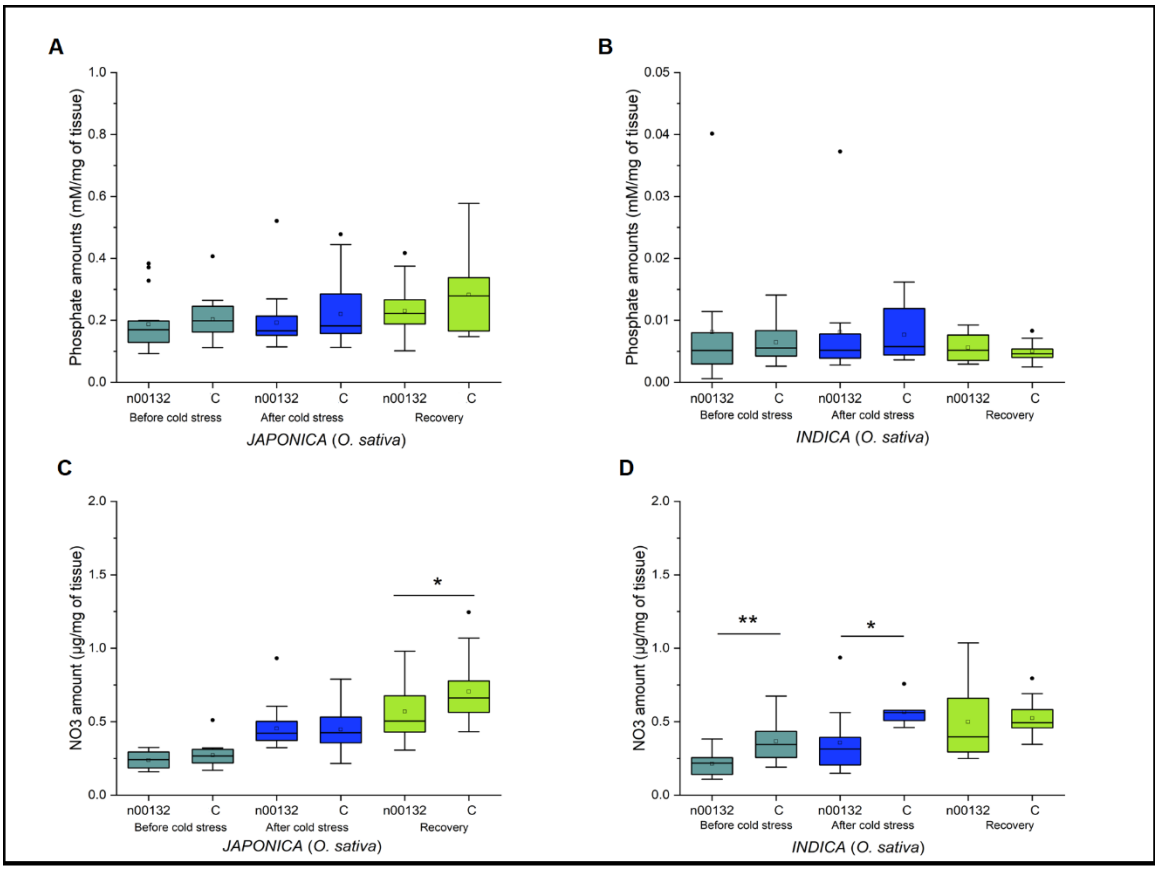

Figure 3.7 Phosphate and nitrate content in rice plants tissues at three time-points: (1) before cold (two-week old plants), (2) after cold (4d4˚C and 4d10˚C for *japonica* and *indica*, respectively), and (3) recovery (one week after cold treatment at warm temperatures). **A & B.** Soluble phosphate amount in *japonica* and *indica* plants. **C & D.** Nitrate amount in *japonica* and *indica* plants. n00132, *P. mosselii* inoculated plants; C, uninoculated control plants. ___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$, **: $p < 0.01$

3.3.4 *P. mosselii* increases the amount of soluble inorganic phosphate in *japonica* plants

To evaluate the influence of *P. mosselii* in converting insoluble phosphate to the soluble form for plants to take up in a hydroponic system, homemade MS media containing $Ca_3(PO_4)_2$ as an insoluble inorganic phosphate was made and uninoculated and inoculated *japonica* plants with *P. mosselii* were grown in this liquid medium. Results showed that inoculated *japonica* plants contained significantly more (50%) soluble inorganic phosphate compared to their uninoculated counterparts (Figure 3.8).

Note: for phosphate measurements in this section, rice seeds were germinated on full strength MS (homemade) and germinated seeds were moved to ¼ MS (homemade) until day 14.

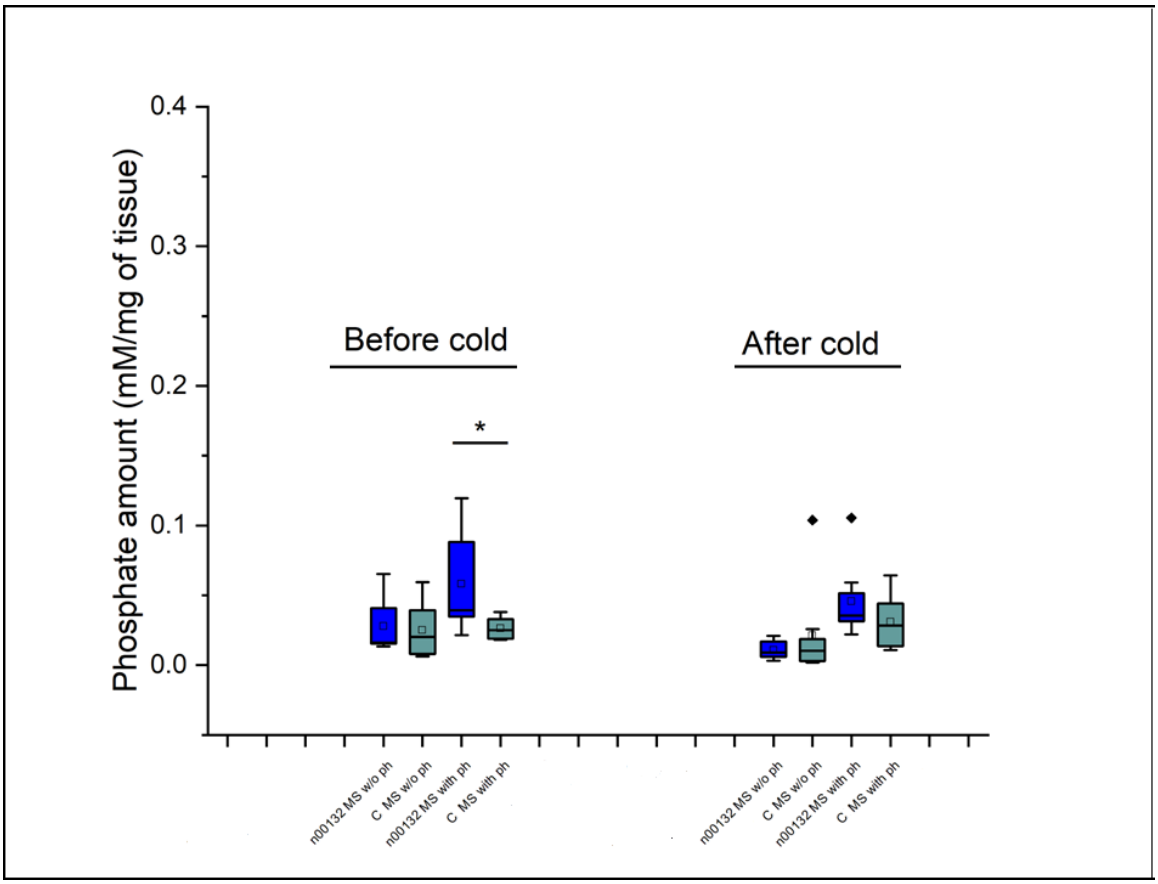

Figure 3.8 *In vivo* phosphate solubilizing activity of *P. mosselii* (n00132) in *japonica* plants. The amounts of soluble phosphate in inoculated *japonica* plants were increased 50% more compared to uninoculated *japonica* plants before cold stress. MS w/o ph = no phosphate in a homemade MS medium, MS with ph = homemade MS medium contained insoluble $Ca_3(PO_4)_2$ instead of soluble KH_2PO_4. ___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$

3.3.5 *P. mosselii* has effects on protein, but no major effects on chlorophyll and sugar levels in plant tissues

The results of measuring protein, chlorophyll, and sugar amount (Figure 3.9) in *P. mosselii* inoculated *japonica* and *indica* plants compared to uninoculated plants showed that only uninoculated *japonica* plants after one week of recovery had 25% higher amounts of protein than inoculated control plants ($p < 0.01$) (Figure 3.9A).

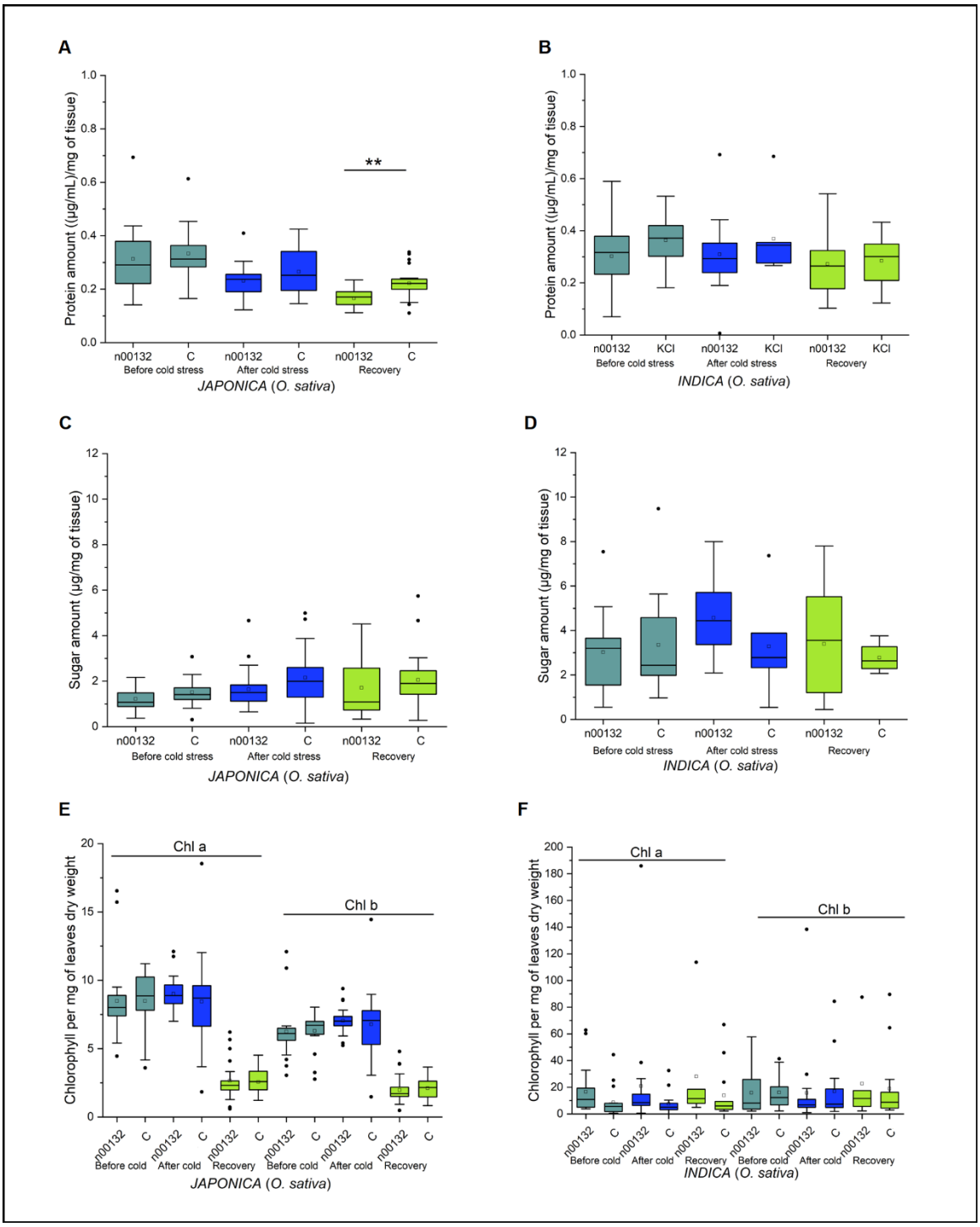

Figure 3.9 Protein, sugar, and chlorophyll content in rice plant tissues at three time-points: (1) before cold (two-week old plants), (2) after cold (4d4˚C and 4d10˚C for *japonica* and *indica*, respectively), and (3) recovery (one week after cold treatment at warm temperatures). **A & B.** Soluble protein amount in *japonica* and *indica* plants. **C & D.** Sugar amount in *japonica* and *indica* plants. **E & F.** Chlorophyll a and b amount in *japonica* and *indica* plants. ___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: **: $p < 0.01$

3.3.6 *P. mosselii* differentially affects proline and reduced glutathione levels in plant tissues

Results of proline and reduced glutathione measurements in *P. mosselii* inoculated and uninoculated plant tissues showed that right before the cold stress treatment, inoculated two-weeks-old *japonica* plants had higher amounts of proline (48% more than uninoculated *japonica* plants) compared to uninoculated plants and *indica* plants had higher reduced glutathione (65% more than uninoculated *indica* plants) compared to uninoculated plants (Figure 3.10A & D). These two compounds increased in inoculated plant tissues before cold stress, and this could be related to the priming effect that *P. mosselii* has on rice plants, which prepares plants for undesirable conditions.

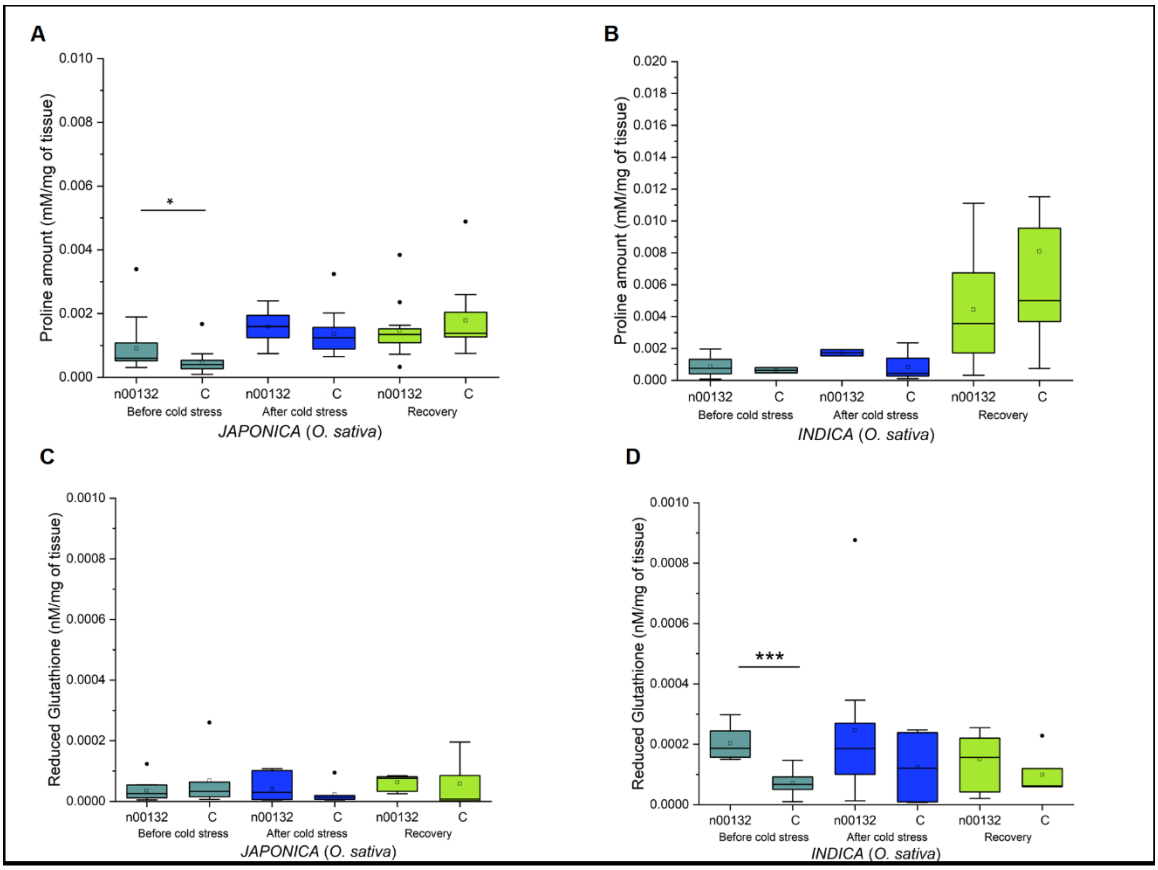

Figure 3.10 Proline and reduced glutathione amount in rice plants tissues at three time-points: (1) before cold (two-week old plants), (2) after cold (4d4˚C and 4d10˚C for *japonica* and *indica*, respectively), and (3) recovery (one week after cold treatment at warm temperatures). **A & B.** Proline amount in *japonica* and *indica* plants. **C & D.** Reduced glutathione amount in *japonica* and *indica* plants. ___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

3.3.7 *P. mosselii* influences ROS levels in inoculated rice plants

Nitro blue tetrazolium (NBT) staining is a method to quantify reactive oxygen species (ROS) levels in cells of living organisms (Shi et al., 2020). Results of NBT staining from *japonica* leaf tissues showed no difference ($p > 0.05$) in ROS levels between inoculated and uninoculated plants before cold stress, although there was a trend for higher levels in inoculated plants (Figure 3.11A & B). There was also no difference ($p > 0.05$) in ROS levels in *indica* and *japonica* leaf tissues between inoculated and uninoculated plants right after cold stress, although there was also a trend for higher levels in control plants (Figure 3.11A & B). However, in both *indica* and *japonica* plants, there was a significant increase ($p < 0.01$) in ROS levels in uninoculated control plants compared to *P. mosselii* inoculated plants 3 days into the recovery period (Figure 3.11A & B, Figure 3.12).

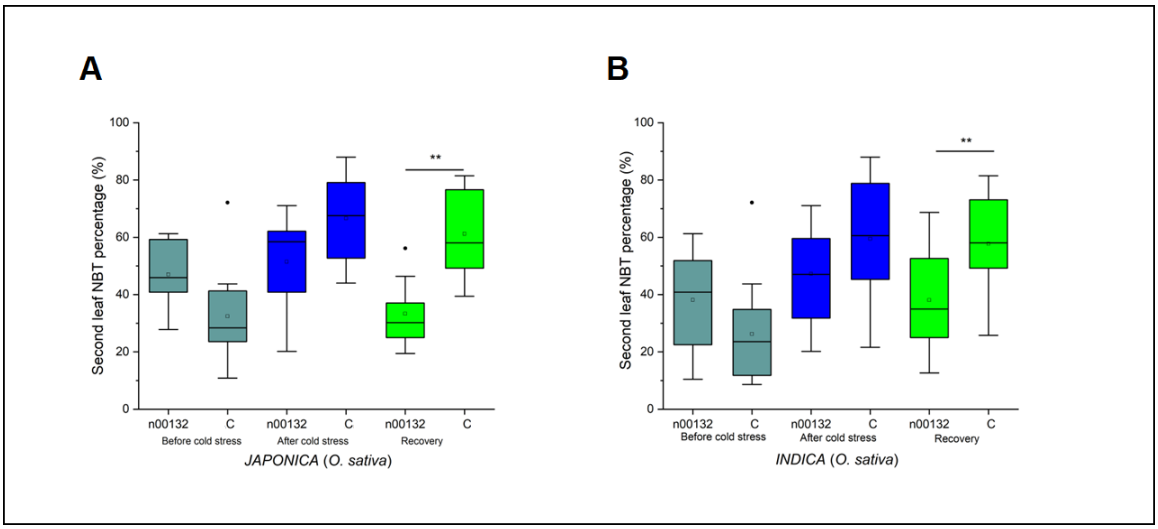

Figure 3.11 ROS levels in rice second leaf tissues detected via NBT staining at three time-points: (1) before cold (two-week old plants), (2) after cold (4d4˚C and 4d10˚C for *japonica* and *indica*, respectively), and (3) recovery (3 days after cold treatment at warm temperatures). **A.** ROS levels in *japonica* plants. **B.** ROS levels in *indica* plants.___= Median line, □ = Mean, ● = Outliers. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$, **: $p < 0.01$, $p < 0.001$

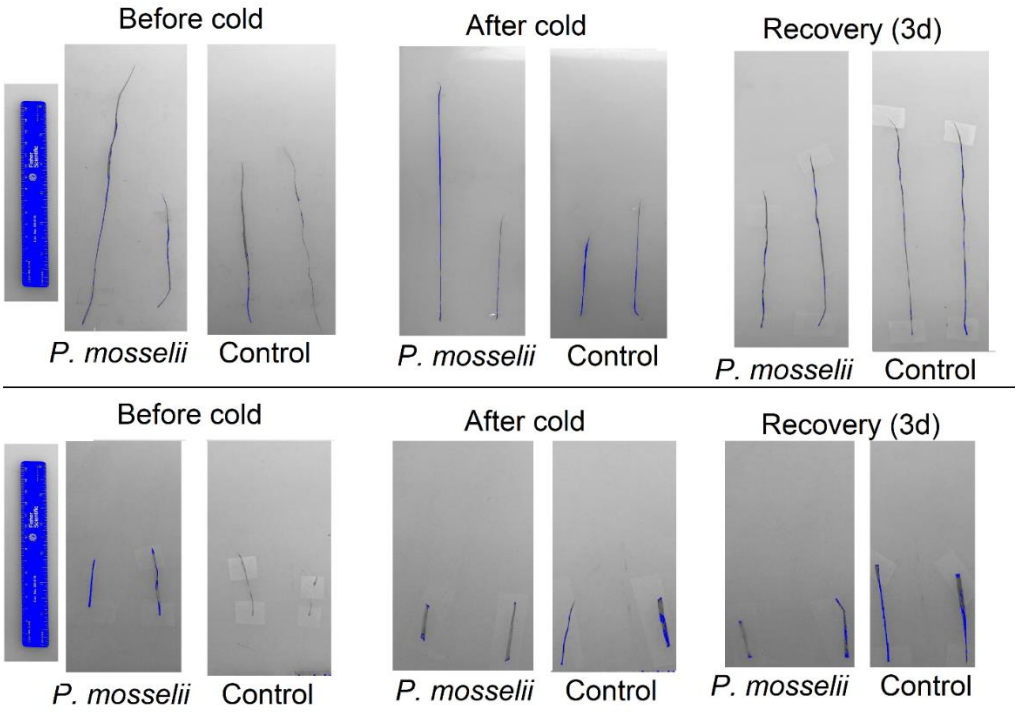

Figure 3.12 Nitro Blue Tetrazolium (NBT) Staining of inoculated *japonica* (upper row) and *indica* (bottom row) leaves with *Pseudomonas mosselii* compared to uninoculated control leaves. Both *japonica* and *indica* plants followed a similar pattern, where control leaves contained more ROS (more blue parts) compared to inoculated leaves during the recovery period (day 3). These pictures were analyzed with ImageJ software.

3.3.8 *P. mosselii* affects transcript levels of proline and glutathione synthesis genes in rice plants

To correlate proline and reduced glutathione activity levels before cold stress (Figure 3.8) with transcript levels of proline and reduced glutathione synthesis genes in *japonica* and *indica* plants, respectively, qPCR analyses were done on *P. mosselii* inoculated and uninoculated control plants. The results showed that transcript levels for proline synthesis genes *OsP5CR* and *OsPRODH* were higher in inoculated than uninoculated *japonica* plants. Specifically, in inoculated *japonica* plants, transcript levels for the proline synthesis gene *OsP5CR* were higher than transcript levels for the proline degrading gene *OsPRODH*. Moreover, transcript levels for the reduced glutathione synthesis gene *OsGR3* were also higher in inoculated than uninoculated *indica* plants (Figure 3.13).

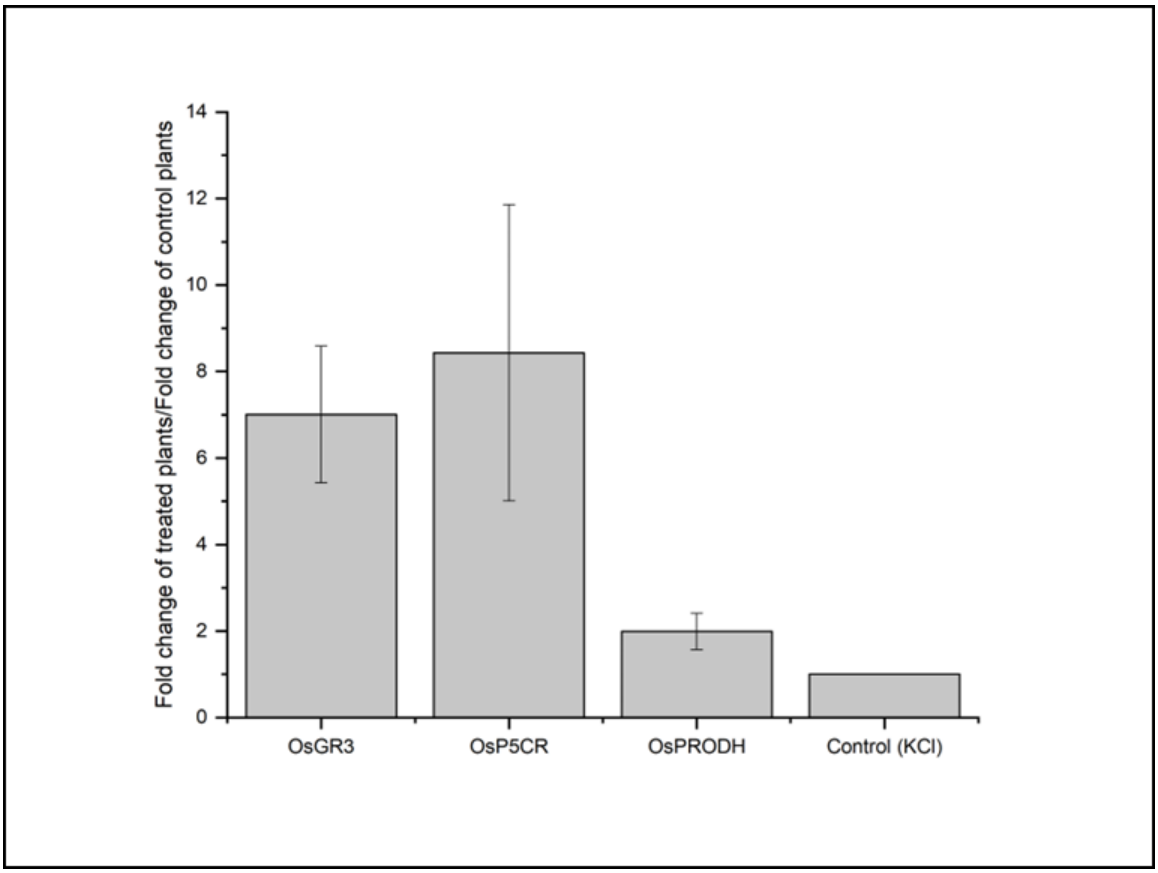

Figure 3.13 Transcript levels of biosynthesis genes for reduced glutathione (*OsGR3*) in *P. mosselii* inoculated *indica* plants, and transcript levels of genes for proline metabolism (*OsP5CR* and *OsPRDOH*) in *P. mosselii* inoculated *japonica* plants, relative to uninoculated controls, in warm conditions (before cold stress). Error bars show standard errors.

3.4 Discussion

To determine whether plant growth promoting bacteria (PGPB) had a positive influence on rice cold tolerance potential, five promising PGPB from our previous research (Maghboli Balasjin et al., 2022) were selected. Among these five bacteria, *Pseudomonas mosselii* had significant plant growth promoting characteristics, such as strong (indole-3-acetic acid (IAA) production, which is an auxin plant hormone that increases plant cell elongation and root growth (Gusmiaty et al. 2019; Maghboli Balasjin et al. 2022). Furthermore, evidence suggests that IAA can act as a stress hormone and improve plant stress tolerance (Shani et al., 2017). This bacterium also produces 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Maghboli Balasjin et al., 2022), which promotes plant growth and stress tolerance (Shikha Gupta & Pandey, 2019). Genome annotation of the isolated *P. mosselii* showed that this bacterium contains genes related to stress response including osmotic and oxidative stresses as well as detoxification

(Maghboli Balasjin et al., 2022). In addition, the bacterium also had the ability to produce some amino acids related to stress tolerance. Proline and glutathione are two compounds that accumulate in plants under stress conditions and work as stress signals which trigger adaptive responses (Hayat et al. 2012;

Noctor et al. 2012). Both proline and reduced glutathione are osmolytes and involved in cellular signaling processes which promote cellular survival when plants are under cold stress. Proline is known to increase ROS in mitochondria through the electron transport chain and, therefore, improves cell survival (Liang et al., 2013). Based on previous observations (Maghboli Balasjin et al., 2022) and the current study, *P. mosselii* was selected as a promising psychrotolerant candidate to improve the performance of rice plants exposed to cold stress.

Results from cold temperature plant growth promoting characterization of *P. mosselii* showed that this bacterium was able to grow and be active in producing IAA, lipase, siderophore, cellulase, hydrolyzing casein and solubilizing phosphate at 4˚C and 10˚C. Measurements of EL and LTSS indicated that *P. mosselii* indeed improved the cold tolerance potential of both *japonica* and *indica* varietal rice plants grown under cold temperature conditions effecting an average of 50% lethality (LT50) in uninoculated plants (Figure 3.1 & 3.2). Because of this positive effect, growth of *P. mosselii* inoculated and uninoculated rice plants was evaluated at the sublethal temperature of 15˚C for the following reasons: First, *P. mosselii* is psychrotrophic and has the ability of growth at 15˚C; and second, 15˚C is a threshold sublethal chilling stress temperature at which rice plants start to experience a reduction in growth compared to warmer temperatures (Danilo Valle Exposito et al., 2022). Temperatures between 0˚C and 15˚C are perceived as cold stress that could influence germination and growth and development of rice plants. Temperatures above 15˚C are critical for proper growth at all stages of rice development (Danilo Valle Exposito et al., 2022). The results showed that *P. mosselii* did not significantly improve shoot or root growth of the *japonica* rice plants at 15˚C, however, compared to uninoculated control plants, *P. mosselii* inoculated *indica* plants had a significant improvement in shoot growth between day 5 and 10 of the recovery period. Because *P. mosselii* was isolated from *indica* leaves and it was previously shown that the genotype of rice plants influenced plant-microbe interactions and that not all PGPB responded similarly to different plant taxa (Maghboli Balasjin et al., 2022), it is interesting to note that *P. mosselii* improved shoot growth after exposure to 15˚C only of *indica* plants, that is, the same rice variety from which it was originally obtained.

It moreover makes sense that *P. mosselii* only improves growth of the *indica* varietal group, because it was previously shown that low temperature seedling survivability (LTSS) and electrolyte leakage (EL) were not negatively affected at 16˚C in the *japonica* varietal group while 16˚C starts to affect LTSS and EL in the *indica* varietal group (Shimoyama et al., 2020). However, these results also showed that *P. mosselii* generally did not improve shoot and root growth at 15˚C, making it unlikely that its positive effect on cold tolerance potential at lower chilling temperatures was due to growth stimulation.

To identify other potential mechanisms for the ability of *P. mosselii* to improve the cold tolerance potential of rice plants at lethal chilling temperatures, plant physiological and molecular assays based on the bacterium's metabolic performances and genomic sequence were performed. Nitrogen and phosphate are the two important macronutrients that increase the photosynthesis efficiency and cold resistance of plants (Gusta et al. 1999; Yulianti and Rakhmawati 2017). Several PGPB can solubilize phosphate and fix nitrogen in inoculated *japonica* plants (Bianco et al., 2021), including *P. mosselii* (Maghboli Balasjin et al., 2022), which facilitates phosphate and nitrogen uptake in plants. According to the results, *P. mosselii* had the ability to convert insoluble $(Ca_3PO_4)_2$ to soluble phosphate and increase the amount of soluble inorganic phosphate in *japonica* rice plants before cold stress (Figure 3.5). As mentioned earlier, soluble phosphate improves cold tolerance in plants by reversing sucrose synthesis inhibition and increasing photosynthesis rate (Hurry et al., 2000). However, the data from our current study did not show an increase in either nitrogen or phosphate in tissues, when liquid ¼ MS (commercial-ready to use powder, pH 5.8) was added to plants on day 10, from either subspecies when inoculated compared to uninoculated plants (Figure 3.5), ruling out phosphate and nitrate uptake as an effective cold tolerance response mechanism in *P. mosselii* inoculated plants.

Significant overall changes in protein levels involving post-transcriptional and post-translational alterations are often used by plants to respond to environmental stress conditions such as cold temperatures (Thapa & Shrestha, 2022). In addition to protein levels, sugar and chlorophyll contents in plant tissues play important roles in stress tolerance responses (Syamsia et al. 2018; Jeandet et al. 2022). Sugars play roles in different phases of the plant life cycle and act as compatible osmolytes and can scavenge ROS in plant tissues under abiotic stress (Jeandet et al., 2022). Under stress conditions, the total content of chlorophyll (Chlorophyll a and b) generally decreases, indicating that the photosynthetic performance of stressed plants

does not act properly (Sherin et al., 2022). It was previously shown that some PGPB stimulate genes in plants that express defense-related proteins and enzymes involved in sugar and chlorophyll biosynthesis (Abdelaal et al., 2021). However, our experiments showed no increase in protein, chlorophyll, or sugar contents in inoculated compared to uninoculated rice plants, ruling those factors out as an effective cold tolerance response mechanism in *P. mosselii* inoculated plants.

Interestingly, in some cases we observed higher amounts of nitrate and total proteins in uninoculated control than *P. mosselii* inoculated rice plants. Specifically, uninoculated *japonica* control plants had significantly higher nitrate (Figure 3.4C) and protein (Figure 4.6A) levels than inoculated plants after 7 days of recovery, while uninoculated *indica* control plants had significantly higher nitrate levels than inoculated plants before and after cold stress (Figure 3.4D). We previously showed that bacterial behavior inside rice tissues is dependent on plant genotype (Maghboli Balasjin et al., 2022). One explanation for the different amount of nitrate at different time-points between *indica* and *japonica* is that *P. mosselii* acts differently in the cold sensitive *indica* and cold tolerant *japonica* subspecies, which have different genomic structures and different characteristics under stress conditions (Liu et al. 2018; Shimoyama et al. 2020). *P. mosselii* might repress *japonica* genes related to nitrogen uptake during the recovery period, which correlates ($p < 0.05$) with the general downregulation of total protein amounts in *japonica* plants during cold and recovery (Figure 3.6A). A general reduction of protein levels during cold and recovery is not observed in *indica* plants (Figure 3.6B), therefore, *P. mosselii* might reduce nitrate uptake in inoculated *indica* plants as part of maintaining homeostasis of proteins and amino acids necessary for their survival under stress conditions (Masclaux-Daubresse et al., 2010).

Other factors that positively regulate plant stress tolerance response mechanisms are the amino acid proline and the thiol containing tripeptide glutathione (Sneha Gupta et al., 2021). When plants are under stress, ROS are produced in plant tissues, which can damage the plant cells through peroxidation (Hayat et al. 2012; Phan and Schläppi 2021). Proline and glutathione are able to scavenge ROS. It is known that under cold temperature, proline accumulates in plant cells to protect them from further damage (Hayat et al., 2012). Reduced glutathione is an antioxidant that defends plant cells against undesirable environmental conditions (Kasote et al., 2015). Proline increases mitochondrial ROS through the electron transport chain. When plants are under stress, proline accumulates in plant cells, which involves regulating

P5CR and *PRODH* transcripts (Liang et al., 2013). The results of proline and reduced glutathione measurements in the current study revealed that *P. mosselii* inoculated *japonica* plants had higher amounts of proline than uninoculated control plants, while *P. mosselii* inoculated *indica* plants had higher amounts of reduced glutathione than uninoculated control plants before cold stress treatment (Figure 3.7). These results are compatible with *P. mosselii* genome annotations from our previous study showing that the *P. mosselii* genome contains genes related to proline and glutathione synthesis (Maghboli Balasjin et al., 2022) and raises the possibility that those molecules are transmitted from bacteria to plant cells, which needs to be addressed in future studies. Interestingly, in addition to "Proline, 4-hydroxyproline uptake and utilization" genes, *P. mosselii* contains genes for "Proline synthesis", which means that alternatively, this bacterium might modulate proline production in plant cells. Plant-bacteria specificity has been studied for many years and there is evidence showing this specificity causes several plant/bacterial cellular responses ("Induced Mutations - A Tool In Plant Research," 1981). Taken together, we conclude that *P. mosselii* might prepare *japonica* and *indica* plants at warm temperatures to respond quickly to cold stress conditions by increasing proline and reduced glutathione levels, respectively, which can be considered a "priming" effect (Aranega-Bou et al., 2014). This priming effect might be transduced by the observed trend for higher ROS levels in *P. mosselii* inoculated *japonica* and *indica* than uninoculated control plants before cold stress (Figure 8), resulting in higher proline and reduced glutathione levels, because it was previously shown that a certain level of ROS can activate numerous stress response pathways (Dreyer & Dietz, 2018).

To determine whether *P. mosselii* influenced plant gene expression regulating proline and reduced glutathione synthesis, qPCR analyses (Figure 3.9) were done using primers specific for proline and reduced glutathione synthesis pathways in rice plants (Table 3.3). As expected from proline level analysis (Figure 7a), before cold stress, transcripts for rice proline synthesis genes pyrroline-5-carboxylate reductase (*OsP5CR*) and proline dehydrogenase (*OsPRODH*) increased almost 8-fold and 2-fold, respectively, in inoculated *japonica* plants compared to control plants. PRODH stimulates the Pro/P5C cycle through converting proline into Δ^1 pyrroline-5-carboxylate (P5C) while P5CR acts in the opposite direction and converts P5C into proline (Monteoliva et al., 2014). When plants are under cold stress, dehydration of plant cells can occur, causing damage to cell membranes (S. K. Yadav, 2009). Therefore, proline synthesis increases in dehydrating plant cells, while its catabolism decreases, thus helping plants to protect their cells

from dehydration stress (Monteoliva et al., 2014). Our results showed that transcripts levels of P5CR (which is related to proline synthesis) were higher than those of PRODH (which is related to proline catabolism), indicating that proline synthesis in *P. mosselii* inoculated *japonica* plants was higher than proline degeneration.

These results are compatible with measured ROS levels (based on NBT staining) in leaves of *P. mosselii* inoculated and uninoculated control plants (Figure 3.8). ROS can act as a signaling molecule to improve plant stress tolerance (Phan & Schläppi, 2021), but extra ROS accumulation negatively affects cellular components due to increased production of malondialdehyde (MDA) in membrane lipids (Moraes et al., 2019). Our results showed that after cold stress and during the recovery growth period, higher amounts of ROS were observed in uninoculated than *P. mosselii* inoculated control plants, which correlated with higher EL and lower LTSS levels (Figure 3.1 & 3.2). Proline works as an antioxidant and helps rice plants to fight oxidative damage caused by ROS accumulation. If plants are not under stress, an increased amount of proline is beneficial, because it prepares them to face undesirable conditions as part of a priming mechanism (Aranega-Bou et al., 2014). Increased proline levels at warm temperatures in *P. mosselii* inoculated *japonica* plants could act as an initial ROS-scavenging mechanism during both the cold and recovery periods.

A similar pattern for reduced glutathione synthesis gene transcripts was observed in *indica* plants. Glutathione reductase (GR) is one of the enzymes that plants secrete to defend themselves against undesirable environmental conditions (H. Verma et al., 2021). GR is involved in keeping the cellular redox balance of reduced glutathione (GSH) to oxidized glutathione disulfide (GSSG) (Hossain et al., 2017). The genome annotation of *P. mosselii* showed that this bacterium contains genes related to stress response and the glutathione redox cycle. It has been previously shown that PGPB with oxidative stress response genes are able to stimulate plant defense mechanisms through scavenging excess amounts of ROS in plants (Khanna et al., 2019). In this study we analyzed transcript levels for the rice GR gene *OsGR3* in *P. mosselii* inoculated and uninoculated control *indica* plants at warm temperatures to correlate transcript levels (Figure 3.8) with reduced glutathione levels (Figure 3.7D). Glutathione production in plants starts in plastids when glutamic acid and cysteine bind and form γ-glutamylcysteine. Next, in plastids or the cytosol, γ-glutamylcysteine and glycine bind, and form reduced glutathione (GSH) through the activity of GSH

synthase. In this step, GSH scavenges excess amounts of ROS and is converted to the disulfide form GSSG. The oxidized GSSG is converted to reduced GSH through glutathione reductase (Hasanuzzaman et al., 2019). Based on our qPCR results, transcript levels of *OsGR3* in inoculated *indica* plants increased almost 7-fold compared to uninoculated control plants (Figure 3.9), correlating well with GSH levels (Figure 3.6D). As *indica* plants went through cold stress and recovery growth periods, ROS levels kept increasing in uninoculated control plants compared to inoculated plants. This suggests that increased amounts of reduced glutathione in *P. mosselii* inoculated *indica* plants before cold stress had a positive priming effect to help them fight the subsequent cold stress. Taken together, our qPCR results suggest that increased proline and glutathione levels in *P. mosselii* inoculated plants is due to changes in rice gene expression, however, we cannot rule out yet that *P. mosselii* also secretes those compounds into plant cells, because the bacterium has genes for proline and glutathione biosynthesis.

Taken together, we have shown here that *P. mosselii*, which was isolated from *indica* Carolino 164 varietal leaves, uses different mechanisms (Figure 3.7 & 3.9) to increase the survival and reduce membrane damage of *japonica* Krasnodarskij 3352 and *indica* Carolino 164 varietal plants exposed to lethal cold temperatures (Figure 3.1 & 3.2). According to our previous study (Maghboli Balasjin et al., 2022), PGPB activities are rice genotype dependent and/or PGPB metabolic pathways respond differently depending on what plant genes they are interacting with. In the current study we confirmed our previous observation that *P. mosselii* responded differently and activated different cold tolerance mechanisms in *japonica* Krasnodarskij 3352 and *indica* Carolino 164 varietal plants. Our study provides a new look at plant-microbe interactions in that different PGPB have different effects in different groups and subgroups of rice, suggesting that they benefit rice plants based on specific interactions with the genomes of those rice plants. Based on our previous (Maghboli Balasjin et al., 2022) and current studies, we showed that one specific bacterium has two different positive effects on the two different subspecies of *O. sativa*. To the best of our knowledge, none of related studies have shown the effect of one local rice-associated bacterial endophyte on its own rice plant host and other subgroups of rice plant hosts within the same rice family. However, this study was focused on the influence of one plant growth promoting bacterium with phosphate solubilizing activity in a controlled environment for a short-term on two varieties of *O. sativa* subspecies at a young stage of their life cycle. Further investigations need to address the long-term influence of *P.*

mosselii on these varietal subspecies at an older stage of their life cycle in rice paddies where temperature fluctuations and undesired environmental conditions can occur. Also, it is crucial to understand the interactions of *P. mosselii* with other PGPB as well as other varieties of *O. sativa* subspecies. This further suggests that compared to a single PGPB, a cocktail of compatible PGPB might have a synergistic positive effect on improving rice cold tolerance potential.

3.5 Conclusions

Pseudomonas mosselii is a promising PGPB with priming effects on two members of the two *O. sativa* subspecies to improve cold tolerance of *japonica* and *indica* plants through increasing proline and reduced glutathione, respectively. This shows that plant-microbe interactions are dependent on the genotypes of both plants and microbes.

3.6 Acknowledgements

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Chapter 4: *Pseudomonas mosselii* controls fungal infection and improves survival of *japonica* rice plants after cold stress through different mechanisms

Abstract

Phytopathogens are organisms that attack plants and cause plant diseases that can result in significant economic losses. The pathogens can be more effective when plants are under stress. Asian rice (*Oryza sativa* L.) is a major staple food for half of the world human population and is primarily grown in warm conditions. When rice plants are under cold stress, they can become more vulnerable to diseases. Some plant growth promoting bacteria (PGPB) can alleviate abiotic and biotic stresses in plants through their metabolic and genetic capabilities. It was hypothesized that tropical and subtropical crop species like rice become more vulnerable to pathogen attacks under cold stress conditions. To test this hypothesis, two pathogenic fungi, identified as *Fusarium fujikuroi* and *Talaromyces aurantiacus,* isolated from subspecies *indica* and *japonica* rice plants, respectively, and the isolated PGPB *Pseudomonas mosselii* from *indica* leaf tissue (phyllosphere) were used to explore the bacterium's antagonism against phytopathogens through *in vitro* and *in vivo* antifungal activities. Results from *in vivo* assays indicated that *P. mosselii* made *japonica* plants less vulnerable to both phytopathogens and improved survival of the plants. Results from *in vitro* plate assays showed that live and dead cells of *P. mosselii* inhibited spore germination of *T. aurantiacus,* while only live cells of this bacterium negatively affected spore germination and vegetative growth of *F. fujikuroi*. This showed there could be several mechanisms, such as different secondary metabolites and defense gene upregulation that *P. mosselii* applied to inhibit growth of two different pathogenic fungi and interactions between different microorganisms and plant were genotype dependent.

Keywords: Plant growth promoting bacteria (PGPB), Asian rice (*Oryza sativa* L.), cold stress, phytopathogenic fungi

4.1 Introduction

Plant growth promoting bacteria (PGPB) are beneficial microorganisms that exist in soil environments and benefit plants and crops through direct and indirect mechanisms (Gutiérrez-Santa Ana et al., 2020). Direct mechanisms include the ones that increase plant growth such as phosphorus and nitrogen uptake as well as plant hormone production, while indirect mechanisms mostly involve plant defense mechanisms such as antifungal activity and secondary metabolite production (Maghboli Balasjin et al., 2022). Indirect mechanisms are crucial for plant survival and, therefore, for agricultural crops (Gutiérrez-Santa Ana et al., 2020). Fungal pathogens are the most common plant pathogens (phytopathogens) that compete with PGPB for nutrition and space and attack plants through spore germination and hyphal penetration of plant tissues (Ruiu 2020; Gutiérrez-Santa Ana et al. 2020). Specifically, when plants are under stress, they are more vulnerable and susceptible to diseases. There are different methods such as usage of chemical fertilizers, pesticides, and fungicides to control crop diseases caused by environmental conditions (J. Liu et al., 2022). It is known that these treatments can cause irreversible environmental damage and it is crucial to find sustainable replacements and breed disease-resistant plants (J. Liu et al.,

2022). Therefore, one solution is to utilize PGPB as biofertilizers which will benefit plants and the ecosystem as well (Gutiérrez-Santa Ana et al., 2020).

Among the many different PGPB, there are some with the ability to emit bacterial volatile compounds (BVCs), which can be used as a biocontrol and microbial fertilizers for protecting and improving agricultural crops instead of chemical fertilizers (Gutiérrez-Santa Ana et al., 2020; Nongthombam Olivia et al., 2023). Some PGPB are able to alleviate abiotic and biotic stresses in the plants through their metabolic and genetic capabilities (Jalil & Ansari, 2018). Also, plants can recognize harmful compounds produced by phytopathogens through signaling hormones such as salicylic acid, jasmonic acid, and ethylene (ET) and trigger their own defense mechanisms. This is known as cross talk between plants and microorganisms (Jalil & Ansari, 2018). PGPB that are involved in protecting plants not only can boost the signaling hormones in plants, they are able to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole acetic acid (IAA), which are plant stress hormones, ~~and~~ that alleviate stress conditions for plants (Jalil & Ansari, 2018).

Recently, climate change has become one of the main concerns of farmers and scientists, due to its negative effects on agricultural products through lower soil fertility and plant microbiome alterations (Lladó et al., 2017). In general, climate change, which causes cold and warm extremes, is one of the global threats to agricultural crop production (Martins et al., 2018). When the soil fertility changes, soil microbial structure changes, and microbial diversity decreases. Consequently, soil productivity will decrease through emergence of plant pathogens (Classen et al., 2015). Plant pathogens (phytopathogens) include different bacteria and fungi within the soil environment (Martins et al., 2018; Jana et al., 2022). These phytopathogens attack plants and cause different plant diseases resulting in significant economic losses (Martins et al., 2018).

Rice (*Oryza sativa* L.) is a major staple food for half of the world human population (McCouch et al., 2016). It is estimated that by the year 2050, the world's population will reach 9.1 billion and therefore agricultural crop production needs to increase (Lastochkina et al., 2019). Rice plants are mostly grown in tropical and subtropical areas, therefore these types of crops are not able to grow properly in non-freezing low (chilling) temperatures due to lack of cold acclimation mechanisms (Sanghera et al., 2011). In these conditions, rice plants use some internal mechanisms and strategies to survive such as specific gene

expression regulation (Yan et al., 2006). However, the length of time cold temperature is imposed is critical for plants to initiate the activation of their immune system and very little is known about the relationship between the length of cold stress and plants' immune activation (Wu et al., 2019). In general, *O. sativa* contains two main subspecies, *indica* and *japonica*, which are significantly different in terms of chilling tolerance (Guo et al., 2018). *Japonica* is more cold tolerant compared to *indica* varieties, because of the artificial selection of single-nucleotide polymorphisms (SNPs) in cold tolerance genes such as *COLD1* (Liu et al., 2018). Under chilling temperatures, *indica* and *japonica* rice plants reveal significantly divergent amounts of reactive oxygen species (ROS) and antioxidant metabolites (Guo et al., 2018).

In this study, we isolated two phytopathogenic fungi identified as *Talaromyces aurantiacus* and *Fusarium fujikuroi* from *japonica* and *indica* varietal rice plants, respectively. Recently, it was shown that *Pseudomonas mosselii*, which was isolated from *indica* leaf tissue (phyllosphere) (Maghboli Balasjin et al., 2022), improved cold tolerance of both *indica* and *japonica* rice plants through increasing reduced glutathione and proline, respectively (Maghboli Balasjin et al., in review; Chapter 3). In addition to these compounds, which are antioxidants and help the plants, we hypothesized that *P. mosselii* can control fungal infections and improve survival of rice plants after cold stress through anti-pathogen activities. To test this hypothesis, both *in vivo* and *in vitro* assays were conducted to understand antifungal activities of *P. mosselii*. Evaluating the effect of *P. mosselii* on cold tolerance of *O. sativa* through antifungal activity of this bacterium is novel. This study determined the influence of *P. mosselii* on rice plants when they are under both abiotic and biotic stresses and evaluated the interactions of this bacterium with different phytopathogens and rice genotypes.

4.2 Materials and Methods

4.2.1 Bacterial isolation from leaves of *indica* rice plants

Pseudomonas mosselii was isolated from two-week old Carolino 164 (GSOR no. 311654), representing *indica* plants. Details of the bacterial isolation can be found in Maghboli Balasjin et al. (2022). Briefly, following the methods of Edwards et al. (2018), rice seeds, Krasnodarskij 3352 (Genetic Stock Oryza [GSOR] no. 311787), representing *japonica* and Carolino 164 (GSOR no. 311654), representing *indica*, were dehulled and surface sterilized using ethanol (70% v/v in dH_2O), hypochlorite sodium (70% v/v in dH_2O), and rinsed with sterile ddH_2O. Then, they were germinated on Murashige-Skoog (MS)

medium at 37˚C for 48 h. Germinated seeds were transferred to an air chamber (12h/12h light/dark cycles 28˚C /25˚C). After 10 d growth in MS medium, seedlings were transplanted into soil pots (details about soil components are mentioned in Maghboli Balasjin et al., 2022). Roots and leaves of the two-week old rice plants were rinsed in sterile water and placed in Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) and sonicated for 30 s at 50-60 Hz three times to ensure all surface microorganisms were dettached. Following the methods of Mano et al. (2007), roots and leaves of the plants were surface sterilized and crushed in a sterile pestle and mortar containing sterile PBS. The liquid ~~of~~ from the crushed roots and leaves was centrifuged (12,074 x g, 10 min) and 100 µL of the supernatant were spread on nutrient agar (NA) (Difco) media and incubated at 30˚C for 48 h. After repeated streaking on NA plates, a total of 140 bacteria, including *P. mosselii*, were isolated.

4.2.2 Fungal isolation from *indica* and *japonica* plants

There are some endophytic microorganisms, known as seed-borne microorganisms, that are found inside plant seeds and are transferred from the mother plant to its progeny (Dumigan & Deyholos, 2022). Among these seed-borne microorganisms, there are some pathogens that inhibit growth and development of PGPB. In this study, we observed and isolated two seed-borne fungi from *indica* and *japonica* plants. The isolated fungi were grown on potato dextrose agar (PDA) (Millipore) in the dark, at room temperature (22˚C - 23˚C) and preserved in 25% glycerol at -80˚C for further usage.

4.2.3 Bacterial whole genome and fungal ITS sequencing

According to our previous study (Maghboli Balasjin et al., 2023, in revision), *Pseudomonas mosselii* isolated from the phyllosphere of the *indica* (Carolina 164) rice variety was chosen for further investigation because of its ability to enhance cold tolerance in rice plants. This bacterium was selected for whole genome sequencing because of its strong phenotypic characterization in plant growth promotion (Maghboli Balasjin et al., 2022). DNA of this bacterium was isolated using the Qiagen DNeasy blood and tissue extraction kit according to manufacturer's instructions and after measuring the concentration of each sample spectrophotometrically (Nanodrop), the DNA sample was sent to the Microbial Genome Sequencing Center (www.migscenter.com) for whole-genome sequencing.

Isolated fungi were grown on PDA plates and based on the GENEWIZ company's instructions, the plates were packaged and sent to the mentioned company (www.genewiz.com) for internal transcribed spacer (ITS) sequencing of nuclear DNA.

4.2.4 *In vitro* antifungal activity

Two different methods for *in vitro* antifungal activity of *Pseudomonas mosselii* were performed: activity against fungal vegetative growth, and activity against fungal spore germination. Vegetative growth antagonism was followed using the methods of Wu et al. (2018) with minor modifications. Briefly, the two fungi were grown on PDA plates separately for 5 d at room temperature (22˚C - 23˚C) in the dark. *P. mosselii* was grown overnight in Luria-Bertani (LB) (Fisher) medium at 30˚C on a shaker (200 rpm). The overnight bacterial culture (OD_{600} = 0.6) was spotted (two 3 µL spots 5 cm from each other) on one set of PDA plates and was incubated at room temperature (22˚C - 23˚C) overnight. A second set of plates was inoculated with four spots added to the PDA plate (each spot had a distance of 3.5 cm from other spots). The next day, fungal colonies from 5-d-old PDA plates containing fungi were inoculated with a sterile loop on the center of the PDA plates containing *P.mosselii* from the day before and incubated in the dark at room temperature (22˚C - 23˚C) for 9 d. Control plates contained only the fungus without any bacteria. The diameter of fungus was measured on days 7, 8 and 9 in both test and control plates.

Because the main goal of this study was to evaluate the antifungal activity of live cells of *P. mosselii* in cold temperatures, the experiment was repeated exactly the same way, but plates were incubated separately at 10˚C and 4˚C (these temperatures were used to cold stress *indica* and *japonica* plants, respectively). Briefly, plates were prepared the same as mentioned above and incubated at room temperature (22˚C - 23˚C) for 5 d. Then, plates were transferred to 10˚C and 4˚C for 8 d. The diameter of the fungus was measured on days 7 and 8 while plates were incubated at cold temperature.

Spore germination antagonism was followed by Dumigan & Deyholos (2022) with minor modifications. Similar to the vegetative growth assay, the two fungi were grown on PDA plates separately for 7 d at room temperature (22˚C - 23˚C) in dark) for spore isolation. *P. mosselii* was grown overnight in Luria-Bertani (LB) medium at 30˚C on a shaker (200 rpm). 1.3 mL of sterile Tween 20 (0.1% v/v in dH_2O) was added to the fungal colonies (5-d-old). The spores were harvested from the fungal colonies with an ethanol flamed sterilized L-shaped spreader through gentle scraping. Spores were collected in sterile tubes

with a 1,000 µL pipetter (approximately 1.8 x 10^7 and 1.3 x 10^7 spores in 1 mL Tween 20 for the two fungi). A 0.5 mL suspension of spores was added and mixed in 1 L of molten PDA at 50˚C. This PDA medium was poured in plates until solidification. Then, two wells were made in each PDA plate with sterile pipettes and 50 µL of *P. mosselii* cell suspension from 6 h, 8 h 24 h and 48 h cultures were added to each hole (each well contained 50 µL of cell suspension of *P. mosselii* from different growth stages (according to the growth curve of *P. mosselii*, OD_{600} for 6 h = 0.2, 8 h = 0.3, 24 h = 0.5, 48 h = 0.4). A similar procedure was repeated with the supernatant and filtered supernatant obtained from *P. mosselii* cultures at different growth stages. The supernatant was prepared by centrifuging bacterial cells (2,415 x g, 10 min) and the filtered supernatant was prepared by filtering this supernatant through a 0.2-µM pore size membrane (Whatman syringe filter). The supernatant contained bacterial cells while the filtered supernatant was free of bacterial cells . There were two sets of control plates, (a) control plates containing 50 µL sterile water and (b) control plates containing 50 µL *Escherichia coli* (*E. coli* strain OP50). This experiment was done using *P. mosselii* at its different growth stages to find the highest antagonism between bacterium and fungus.

Antifungal activity of live cells of *P. mosselii* against spore germination in cold temperatures was tested in the same way as the vegetative growth assay. Plates were incubated at room temperature for 2 d and then transferred to 10˚C and 4˚C for 8 d. Control plates for cold experiments contained 50 µL of sterile water.

Experiments related to room temperature (22˚C - 23˚C) incubation were performed three times, independently. Experiments related to cold temperatures incubation were done twice, independently.

4.2.5 *In vitro* antifungal activity of *P. mosselii* dead cells

As mentioned above, the antifungal activity of *P. mosselii* from different growth stages was tested. It is known that dead cells of bacteria play an important role in stimulating or inhibiting growth of neighboring live microorganisms by releasing amino acids or volatile organic compounds (VOCs) (Ebadzadsahrai et al., 2020; Smakman & Hall, 2022). To test for antifungal activity of dead cells of *P. mosselii*, the tube containing an overnight grown culture was placed in a boiling water bath for 5 min to damage and lyse bacterial cells (heat-treated). 100 µL of the heat treated cell suspension were spread on nutrient agar (NA) and incubated at 30˚C to confirm the bacterial cells were dead. After observing no

growth on NA medium, antifungal plate assays were done exactly the same as above (both first and second method). Control plates contained 50 µL of sterile water. The plates were checked on day 2 after incubation at room temperature (22˚C - 23˚C). This experiment was performed twice for vegetative growth and three times for spore germination, independently. The same experiment was repeated with filtered supernatant of heat treated *P. mosselii* cells.

4.2.6 Secondary metabolites of *P. mosselii* and solvent extraction

To identify potential inhibitory compounds that are produced by *P. mosselii*, a genome analysis was done through antiSMASH (Blin et al., 2021). A Fasta file of the whole genome of *P. mosselii* was uploaded to the antiSMASH website to annotate gene clusters related to secondary metabolite production in *P. mosselii* (Taxonomy ID: 78327).

To extract secondary metabolites, a solvent extraction with ethyl acetate was done following the methods of Alajlani et al. (2016) with minor modifications. Briefly, three flasks each containing 20 mL Luria-Bertani (LB) medium inoculated with a single colony of *P. mosselii* were grown for 48 h at 30˚C. Then, 5 mL of culture from each of two flasks were transferred to 8 conical tubes and centrifuged for 10 min (3214 x g). One tube was used as supernatant only (containing bacterial cells), and the second tube was used as filtered supernatant (bacterial cell-free after using a 0.2-µM pore size membrane Whatman syringe filter). The third flask was placed in boiling water for 5 min to inactivate and kill the bacterial cells. To make sure that bacterial cells were dead, 100 µL of the heat treated culture was spread on LB agar medium and incubated at 30˚C. No growth was observed. 5 mL of the heat treated cells were transferred to four 15 mL conical tubes. To extract secondary metabolites, ethyl acetate was used. From each set of tubes, 15 mL were mixed with 40 mL ethyl acetate and extracted. The organic solvent fraction of each set was placed in a rotary evaporator to obtain the organic extract. As a final step, following the methods of Makuwa & Serepa-Dlamini, (2021) 0.02 g of the organic extract was dissolved in 1mL dimethyl sulfoxide (DMSO) (final concentarion = 20 mg mL^{-1}) and stored at 4˚C for further usage. To test for *in vitro* antifungal activity of the extracted metabolites from the supernatant, filtered cell-free supernatant and heat treated cells were treated as described above. Control plates contained 50 µL of sterile ddH_2O.

4.2.7 Rice seed inoculation with *P. mosselii* and seedling inoculation with fungal spores and evaluation of cold survival of rice plants

Inoculation of rice seeds (both subspecies) was previously described (Maghboli Balasjin et al., 2022). Briefly, rice seeds were dehulled and surface sterilized using 70% (v/v) EtOH (1 min), rinsed three times with sterile water, followed by 70% (v/v) sodium hypoclorite (5 min) and rinsed three times with sterile water. Surface sterilized seeds were transferred to a suspension of 10^8 bacterial CFU in 0.85% (w/v) KCl and placed on a shaker (200 rpm) overnight. Control seeds (uninoculated) were soaked in 0.85% (w/v) KCl. Then, seeds were transferred to full strength solid Murashige-Skoog (MS) medium at 30˚C for 48 h for germination. Germinated seeds were moved to autoclaved magenta boxes, which contained sterile dH_2O, with lids closed, and placed in a growth chamber with cycles of 12 h of light (28˚C) and 12 h of dark (24˚C). Fungal spore inoculation was done following the methods of Thuan et al. (2006), with minor modifications. As described (Dumigan & Deyholos, 2022), 7-d-old fungal cultures on PDA medium were used for spore harvest. 20 mL fungal suspension which contained 5 x 10^4 spores per mL was prepared for each fungus in sterile ddH_2O and used to inoculate 7-d-old rice seedlings. 5 mL of fungal suspension was added to each magenta box containing 6 to 8 rice plants and were spread over leaves and seeds using a 1,000 µL pipette under a laminar flow hood. Magenta boxes were closed and sealed with other autoclaved magenta boxes. This experiment was repeated three more times, independently. In each trial, three magenta boxes containing control plants (without bacterial inoculation) and three magenta boxes containing bacterial inoculated plants were prepared. Out of three control magenta boxes, two were inoculated with the different fungal spores. The same procedure was done for the magenta boxes containing bacterial inoculated plants (Figure 4.1). On day 10, ddH_2O was replaced with autoclaved ¼ Murashige-Skoog (MS) in each magenta box. Plants were grown for 14 d to apply cold stress on them.

Figure 4.1 14-d old bacterial inoculated [red (*indica*) and blue (*japonica*) labels] and uninoculated (green labels, both *indica* and *japonica*) rice plants, which were inoculated with fungal spores on day 7 (two magenta boxes for each bacterial inoculated and uninoculated control plants).

Following our previous study (Chapter 3, Maghboli Balasjin et el., 2023, in revision) and studies by Schläppi et al. (2017), cold stress was applied to two-week old rice where the plants had reached the 2-leaf stage of development. Seedlings (bacterial-fungal inoculated and uninoculated) were transferred to cold temperature growth chambers (12 h/12 h light/dark cycles). To evaluate low temperature seedling suvivability (LTSS), the temperature and length of cold stress was 4d 0˚C and 4d4˚C for *indica* and *japonica*, respectively. This length and temperature exposure for each subspecies lead to survival of approximately half of the plant popoulation (LT50; Chapter 3, Maghboli Balasjin et el., 2023, in revision). As soon as the cold stress was finished, seedlings were put back into a warm temperature growth chamber (12 h/12 h light 28˚C/dark 24˚C cycles) for a three-week recovery growth period. Then, plants were evaluated for LTSS.

4.3 Results

4.3.1 Fungal ITS sequencing

ITS DNA sequencing results identified the two isolates fungi as *Fusarium fujikuroi* and *Talaromyces aurantiacus*. The sequences were deposited at the NCBI, and GenBank accession no. OR103127-OR103128 and OR120327-OR120328, respectively. The sequences were compared with Genbank/Mycobank/nucleotide sequence databases (Mohammadi & Maghboli Balasjin, 2014).

4.3.2 Live cells and unfiltered supernatant of *P. mosselii* negatively affect spore germination and vegetative growth of fungi

Results of *in vitro* antifungal activity against spore germination showed that live cells and unfiltered supernatant (containing some bacterial cells) of *P. mosselii* collected at different bacterial growth stages [late exponential (6 & 8 h), stationary (24 h) and late stationary (48 h) phases] at room temperature were able to inhibit spore germination of *F. fujikuroi* and *T. aurantiacus* (Fig. 4.2A).

In vitro antifungal activity against fungal vegetative growth showed that live cells of *P. mosselii* at room temperature had the ability to inhibit vegetative growth of *F. fujikuroi* (Fig. 4.2B, Fig 4.3 & Table 4.1).

4.3.3 Filtered supernatant of live and dead cells *P. mosselii* had no effect on the two phytopathogens, while unfiltered dead cells had negative effect on spore germination of *T. aurantiacus*

Compared to the presence of live cells, the filtered supernatant free of live and dead bacterial cells had no antifungal activity either for spore germination or vegetative growth (Fig. 4.2C).

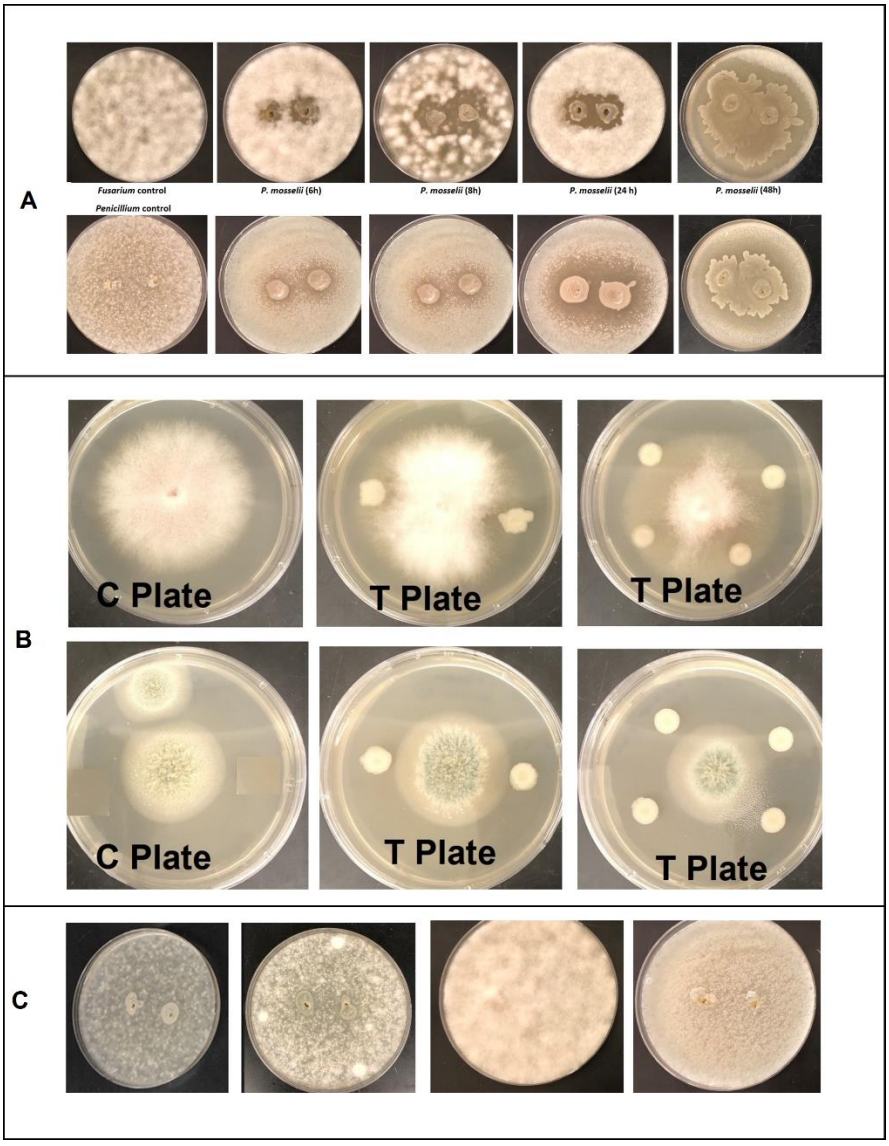

Figure 4.2 Growth inhibition of *F. fujikuroi* and *T. aurantiacus* in presence of *P. mosselii* at room temperature. **A.** Fungal spore germination plate assay. Top row from left to right shows: *F. fujikuroi* control plate containing two holes with 50 µL sterile water; *F. fujikuroi* test plate containing two holes with 50 µL *P. mosselii* live cells that were grown for 6 h, 8 h, 24 h and 48 h. Bottom row shows: *T. aurantiacus* control plate; *T. aurantiacus* test plate containing two holes with 50 µL *P. mosselii* cells that were grown for 6 h, 8 h, 24 h and 48 h. **B.** Fungal vegetative growth stage plate assay. Top row from left to right shows: *F. fujikuroi* control plate; *F. fujikuroi* test plate containing two holes with 3 µL spotted overnight grown *P. mosselii* cells; *F. fujikuroi* test plate containing four holes with 3 µL spotted overnight grown *P. mosselii* cells. Bottom row shows: *T. aurantiacus* control plate; *T. aurantiacus* test plate containing two holes with 3 µL spotted overnight grown *P. mosselii* cells; *T. aurantiacus* test plate containing four holes with 3 µL spotted overnight grown *P. mosselii* cells. **C.** No fungal growth inhibition was observed with the filtered supernatant. From left to right: control plate containing *F. fujikuroi* with two holes containing 50 µL *E. coli* (OP50); control plate containing *T. aurantiacus* with two holes containing 50 µL *E. coli* (OP50;, *F. fujikuroi* with two holes containing 50 µL of filtered supernatant of overnight grown *P. mosselii* cells; and *T. aurantiacus* with two holes containing 50 µL of filtered supernatant of overnight grown *P. mosselii* cells.

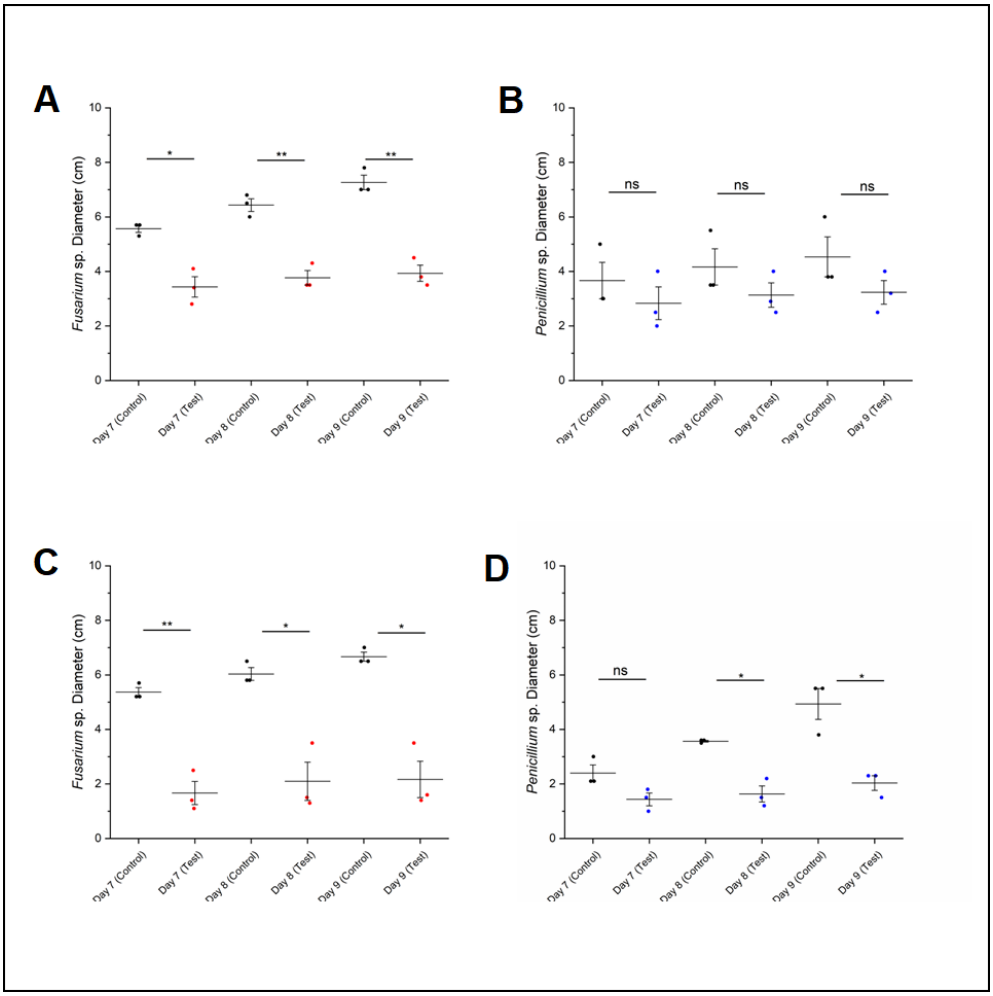

Figure 4.3 Antifungal activity of live cells of *P. mosselii* (fungal vegetative growth plate assay). **A & C.** Fungus diameter when two and four 3 µL of overnight grown *P. mosselii* cells were spotted around *F. fujikuroi* on Days 7, 8, and 9. There was less growth of *F. fujikuroi* on test plates. **B & D.** Fungus diameter when two and four 3 µL of overnight grown *P. mosselii* cells were spotted around *T. aurantiacus* on Day 7, 8, and 9. There was no difference between growth of *T. aurantiacus* on test plates and control plates on Day 7. However, when *T. aurantiacus* was surrounded by four 3 µL of overnight grown *P. mosselii* cells, reduced growth of *T. aurantiacus* was observed on Day 8 & Day 9. Statistical analyses: two-tailed student *t*-test: *: $p < 0.05$

Table 4.1 *In vitro* antifungal activity of *P. mosselii* by adding 3 µL of live cells (2 and 4 spots) on PDA plates surrounding pathogenic fungus. Fungal diameters were measured on test and control plates on days 7, 8, and 9 at room temperature.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	5.3	4.1	Plate 1	3	2
Plate 2	5.7	2.8	Plate 2	3	2.5
Plate 3	5.7	3.4	Plate 3	5	4
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**

F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	6.5	4.3	Plate 1	3.5	2.5
Plate 2	6.1	3.5	Plate 2	3.5	2.9
Plate 3	6.8	3.5	Plate 3	5.5	4
Day 9 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 9 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	7.8	4.5	Plate 1	3.8	2.5
Plate 2	7	3.5	Plate 2	3.8	3.2
Plate 3	7	3.8	Plate 3	6	4
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	5.7	2.5	Plate 1	3	1.8
Plate 2	5.2	1.4	Plate 2	2.1	1.5
Plate 3	5.2	1.1	Plate 3	2.1	1
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	6.5	3.5	Plate 1	3.5	2.2
Plate 2	5.8	1.5	Plate 2	3.6	1.5
Plate 3	5.8	1.3	Plate 3	3.6	1.2
Day 9 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 9 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	7	3.5	Plate 1	3.8	2.3
Plate 2	6.5	1.6	Plate 2	5.5	1.5
Plate 3	6.5	1.4	Plate 3	5.5	2.3

Results indicated that unfiltered dead cells of *P. mosselii* inhibited only spore germination of *T. aurantiacus* and had no effect either spore germination or vegetative growth of *F. fujikuroi* (Figure 4.4A & B, Table 4.2).

Overall, these results showed that the existence of live cells of *P. mosselii* was necessary for inhibition of spore germination and vegetative growth of *F. fujikuroi*. Live and dead cells of *P. mosselii* inhibited spore germination of *T. aurantiacus*. On the other hand, live and dead cells of *P. mosselii* had no effect on vegetative growth of this phytopathogen. Therefore, the data suggest there is more than one mechanism that inhibits growth of these two different phytopathogens.

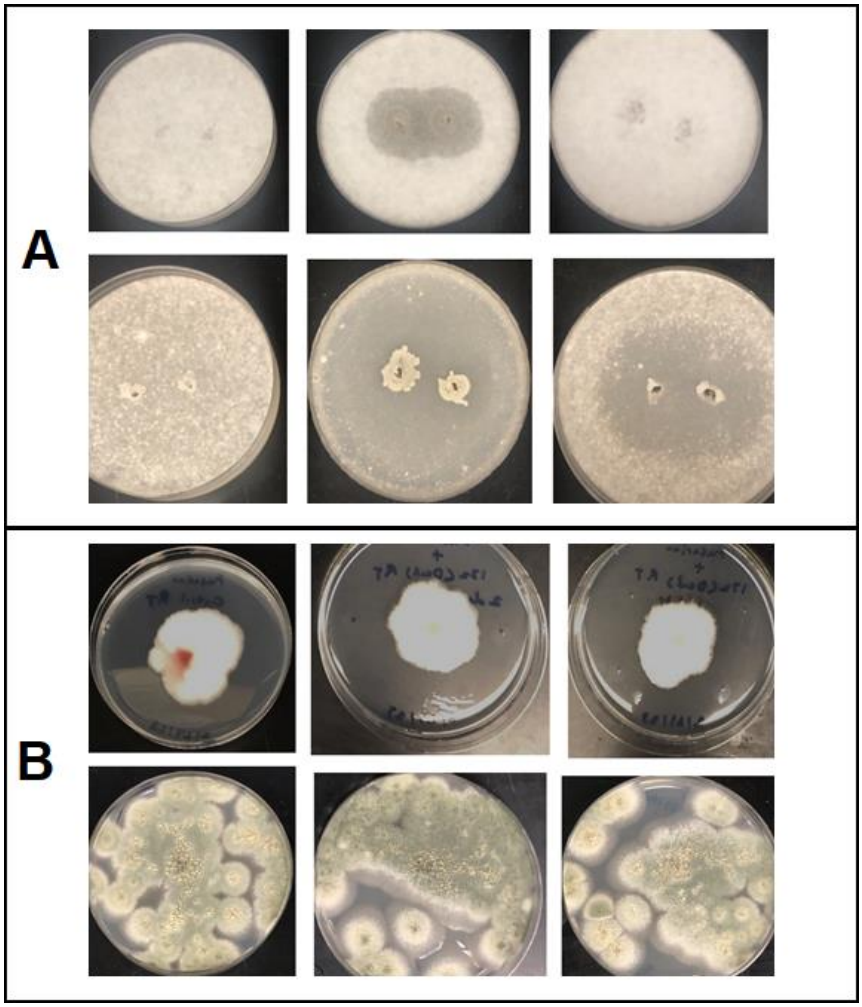

Figure 4.4 Growth inhibition of *F. fujikuroi* and *T. aurantiacus* in presence of *P. mosselii* heat-treated dead cells at room temperature. **A.** Fungal spore germination plate assay. Top row from left to right shows: *F. fujikuroi* control plate containing two holes with 50 µL sterile water; *F. fujikuroi* test plate containing two holes with 50 µL overnight grown *P. mosselii* live cells; *F. fujikuroi* test plate containing two holes with 50 µL heat-treated dead cells of *P. mosselii.* Second row from left to right shows: *T. aurantiacus* control plate containing two holes with 50 µL sterile water; *T. aurantiacus* test plate containing two holes with 50 µL overnight grown *P. mosselii* live cells; *T. aurantiacus* test plate containing two holes with 50 µL heat-treated lysed cells of *P. mosselii.* **B.** Fungal vegetative growth stage plate assay. Top row from left to right shows: *F. fujikuroi* control plate; *F. fujikuroi* test plate containing two holes with 3 µL dead cells of *P. mosselii; F. fujikuroi* test plate containing four holes with 3 µL dead cells of *P. mosselii*. Bottom row shows: *T. aurantiacus* control plate; *T. aurantiacus* test plate containing two holes with 3 µL dead cells of *P. mosselii; T. aurantiacus* test plate containing four holes with 3 µL dead cells of *P. mosselii*.

Table 4.2 *In vitro* antifungal activity of *P. mosselii* by adding 3 µL of dead cells (2 and 4 spots) on PDA plates surrounding pathogenic fungus. Fungal diameters were measured on test and control plates on days 7 and 8 at room temperature.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	4.1	3.2	Plate 1	5	4.9

Plate 2	4.2	3.6	Plate 2	4.6	5.5
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	4.6	3.5	Plate 1	5	6
Plate 2	4.7	4	Plate 2	4.9	7
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	4.1	3.1	Plate 1	5	3
Plate 2	4.2	3	Plate 2	4.6	4
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	4.6	3.5	Plate 1	5	3.3
Plate 2	4.7	3.5	Plate 2	4.9	4

4.3.4 Live and dead cells of *P. mosselii* inhibit spore germination and vegetative growth of fungi when incubated at cold temperatures

Because the main hypothesis of this study is that *P. mosselii* protects rice plants against the two selected phytopathogenic fungi under warm and cold temperatures, the same *in vitro* antifungal plate assays were done under the cold temperatures used to cold stress rice plants (Figure 4.5). For the antifungal spore germination assay, the same results as mentioned above for warm temperatures were observed (Figure 4.5A & B, E & F). Live cells of *P. mosselii* inhibited spore germination of *F. fujikuroi* and *T. aurantiacus* (Figure 4.5A & B) as well as dead cells of *P. mosselii* inhibited *T. aurantiacus* spore germination (Figure 4.5E & F).

For the antifungal vegetative growth inhibition assay, the same results as mentioned above for warm temperatures were observed (Figure 4.5C & D, G & H). Live cells of *P. mosselii* inhibited vegetative growth of *F. fujikuroi* at 10˚C and 4˚C, while they had no effect on *T. aurantiacus* (Figure 4.5C & D). Dead cells of *P. mosselii* had no effect on either phytopathogen (Figure 4.5G & H, Tables 4.3,4.4,4.5,4.6).

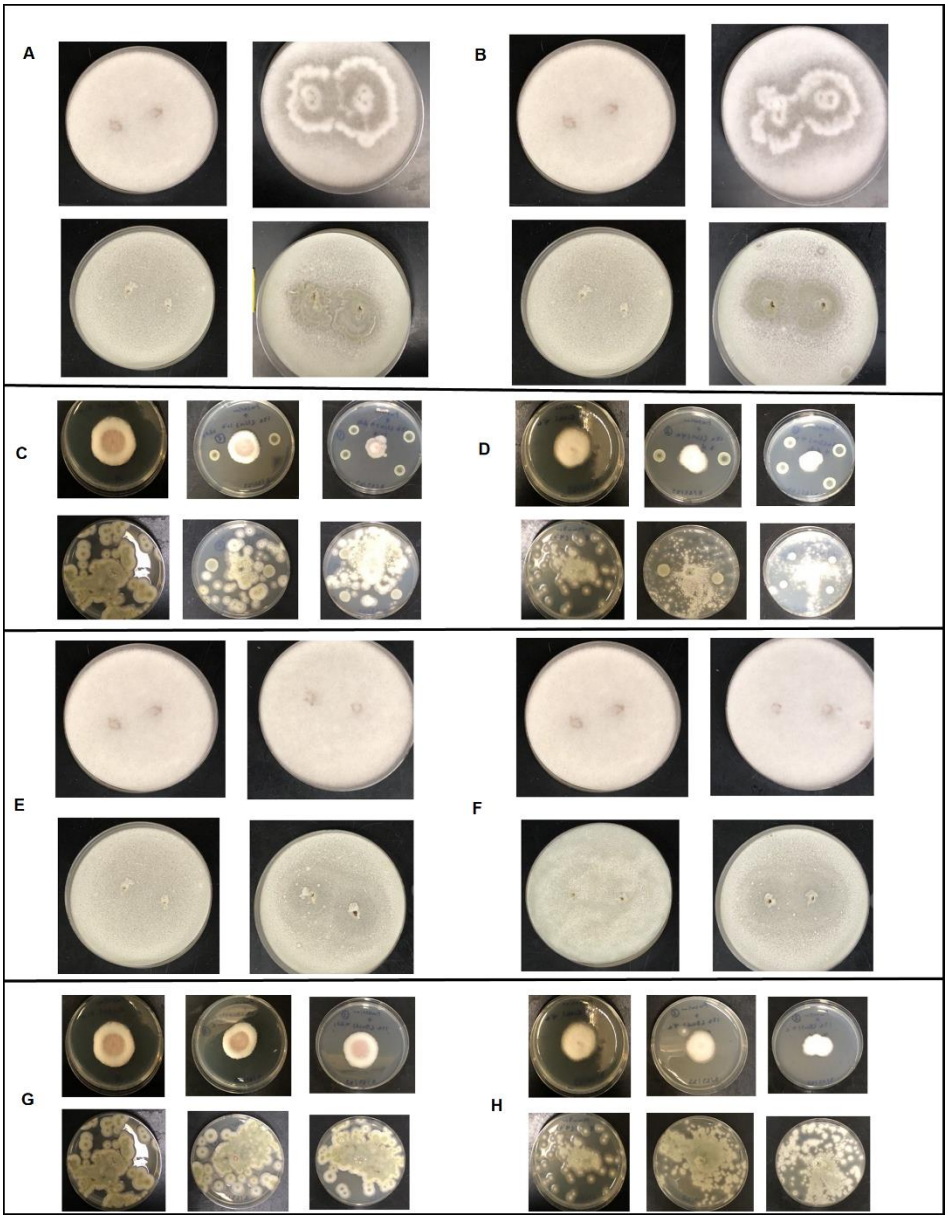

Figure 4.5 *In vitro* antifungal activity at 10˚C and 4˚C. **A.** Fungal spore germination plate assay at 10˚C. Top row: *F. fujikuroi* control plate containing two holes with 50 µL sterile water; and *F. fujikuroi* test plate containing two holes with 50 µL overnight grown live *P. mosselii* cells. Bottom row: same order as the top row, but for *T. aurantiacus.* **B.** Fungal spore germination plate assay at 4˚C. The order for top and bottom rows are the same as 10˚C. **C.** Fungal vegetative growth plate assay at 10˚C. Top row: *F. fujikuroi* control plate; *F. fujikuroi* test plate containing two holes with 3 µL overnight grown live *P. mosselii* cells; and *F. fujikuroi* test plate containing four holes with 3 µL overnight grown live *P. mosselii* cells. Bottom row: same order as the top row, but for *T. aurantiacus.* **D.** Fungal vegetative growth plate assay at 4˚C. The order for top and bottom rows are the same as 10˚C. **E & F.** The same experiment as described for **A & B** was done but for dead cells of *P. mosselii*. **G & H.** The same experiment as described for **C & D** was done but for dead cells of *P. mosselii*.

Table 4.3 *In vitro* antifungal activity of live cells of *P. mosselii* against vegetative growth of the two phytopathogens. Fungal diameters were measured on test and control plates on days 7 and 8 at 10˚C.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.6	2.9	Plate 1	4	2
Plate 2	3	2.6	Plate 2	3.6	2.7
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.7	2.9	Plate 1	4	2.1
Plate 2	3	2.9	Plate 2	3.9	3.4
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.6	1.9	Plate 1	4	2.5
Plate 2	3	2.3	Plate 2	3.6	5
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.7	2	Plate 1	4	3
Plate 2	3	2.3	Plate 2	3.9	5

Table 4.4 *In vitro* antifungal activity of live cells of *P. mosselii* against vegetative growth of the two phytopathogens. Fungal diameters were measured on test and control plates on days 7 and 8 at 4˚C.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.3	2.5	Plate 1	3.3	3.5
Plate 2	2.4	2.6	Plate 2	2.8	2
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.4	2.5	Plate 1	3.5	3.5
Plate 2	2.5	2.8	Plate 2	2.9	2.1
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.3	2.3	Plate 1	3.3	1.6
Plate 2	2.4	2.2	Plate 2	2.8	2.5
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.4	2.3	Plate 1	3.5	1.6
Plate 2	2.5	2.2	Plate 2	2.9	2.5

Table 4.5 *In vitro* antifungal activity of dead cells of *P. mosselii* against vegetative growth of the two phytopathogens. Fungal diameters were measured on test and control plates on days 7 and 8 at 10˚C.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.6	3.6	Plate 1	4	4.8
Plate 2	3	3.3	Plate 2	3.6	5.1
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.7	3.9	Plate 1	4	5
Plate 2	3	3.1	Plate 2	3.9	4.8
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.6	3.3	Plate 1	4	3.7
Plate 2	3	2.8	Plate 2	3.6	3.2
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.7	3.6	Plate 1	4	4
Plate 2	3	2.9	Plate 2	3.9	3.4

Table 4.6 *In vitro* antifungal activity of dead cells of *P. mosselii* against vegetative growth of the two phytopathogens. Fungal diameters were measured on test and control plates on days 7 and 8 at 4˚C.

Day 7 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.3	2	Plate 1	3.3	3.8
Plate 2	2.4	2.9	Plate 2	2.8	2
Day 8 (2 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (2 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.4	2	Plate 1	3.5	4
Plate 2	2.5	3	Plate 2	2.9	2
Day 7 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 7 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.3	3.1	Plate 1	3.3	3.5
Plate 2	2.4	3	Plate 2	2.8	3
Day 8 (4 spotted)	**Diameter (cm)**	**Diameter (cm)**	**Day 8 (4 spotted)**	**Diameter (cm)**	**Diameter (cm)**
F. fujikuroi	**Control plate**	**Test plate**	***T. aurantiacus***	**Control plate**	**Test plate**
Plate 1	3.4	3.2	Plate 1	3.5	3.5
Plate 2	2.5	3.2	Plate 2	2.9	3

4.3.5 Analysis of the *P. mosselii* genome identifies genes for secondary metabolites associated with antifungal activities

Based on antiSMASH genome analysis, *P. mosselii* has genes that might produce secondary metabolites with known antibiotic and antifungal activities (Table 4.7). Among these compounds, siderophores with a peptide alkaloid structure are known to have strong antifungal effects.

4.3.6 Solvent extractions from *P. mosselii* had no effect on the two phytopathogens

Solvent extraction showed no effect on inhibiting spore germination of either phytopathogens by live or dead cells of *P. mosselii* as well as the filtered supernatant of *P. mosselii*. However, it was found that DMSO used to solubilize extracted compounds had a negative effect by itself on spore germination of *T. aurantiacus*, because control plates with pure DMSO and *T. aurantiacus* showed the same pattern as test plates containing extracted solvents and *T. aurantiacus* (Figure 4.6).

Table 4.7 Secondary metabolite operon regions of *Pseudomonas mosselii* identified using strictness 'relaxed'. NRPS = Non-ribosomal peptide synthetase cluster; T3PKS = Type III PKS (Polyketide synthase); NAGGN = N-acetylglutaminylglutamine amide; RiPP-like = Other unspecified ribosomally synthesized and post-translationally modified peptide product (RiPP).

Region	Type	From	To	Most similar known cluster		Similarity
Region 9.1	NRPS	53,817	106,764	pyoverdin	NRP	11%
Region 10.1	RiPP-like,PpyS-KS	81,747	107,962	pseudopyronine A / pseudopyronine B	Other:Fatty acid	75%
Region 19.1	NRPS	26,161	72,499	putisolvin	NRP	50%
Region 21.1	NAGGN	49,406	64,311			
Region 26.1	RiPP-like	18,450	29,283			
Region 36.1	redox-cofactor	34,847	55,376	lankacidin C	NRP + Polyketide	13%
Region 50.1	NRPS	1	35,874	pseudomonine	NRP	100%

Region 60.1	NRPS	1	30,861	tolaasin I / tolaasin F	NRP:Lipopeptide	50%
Region 67.1	NRPS	1	23,660	entolysin	NRP	43%
Region 73.1	ranthipeptide	1	15,080	pyoverdin	NRP	4%
Region 80.1	NRPS-like	1	17,128	fragin	NRP	37%
Region 81.1	NRPS	1	16,915	pyoverdin	NRP	11%
Region 87.1	T3PKS	1	14,738			
Region 91.1	NRPS	1	12,589	crochelin A	NRP + Polyketide	11%
Region 105.1	NRPS	1	4,911	pyoverdin	NRP	3%
Region 107.1	NRPS	1	4,374			

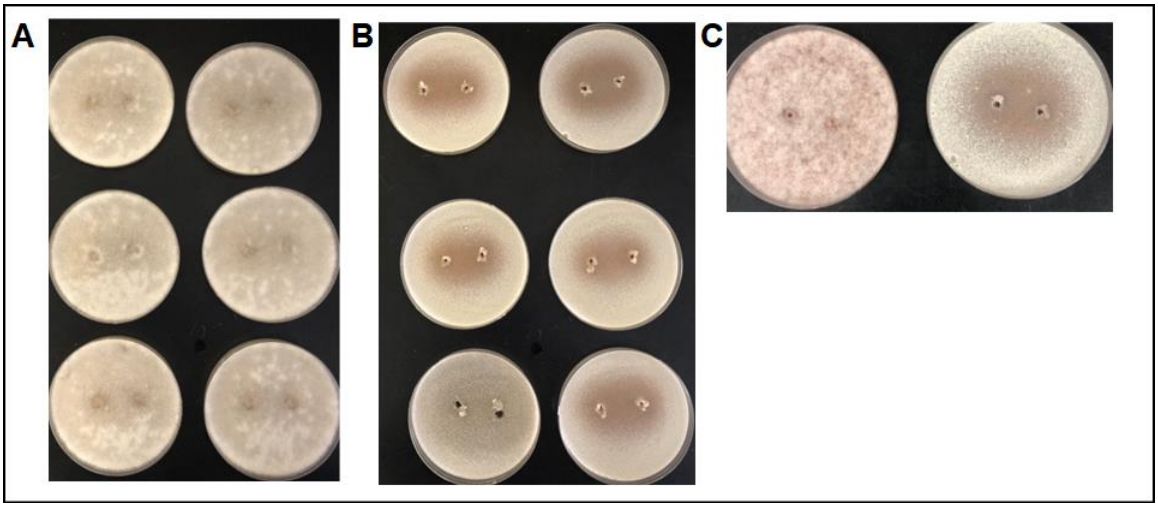

Figure 4.6 Test for antifungal activity against spore germination using organic solvent extractions of *P. mosselii* cells. **A.** *Fusarium* sp. plates. Top row plates were inoculated with organic solvent extraction from bacterial cell-free (filtered) supernatant; middle row was inoculated with organic solvent extraction from non-filtered bacterial supernatant; and bottom row was inoculated with organic solvent extraction from lysed bacterial cells. No growth inhibition of *F. fujikuroi* was observed. **B.** *T. aurantiacus* plates. The order of plates is the same as for *F. fujikuroi* plates. **C.** Control plates with pure DMSO. From left to right: *F. fujikuroi* and *T. aurantiacus.* Growth inhibition of *T. aurantiacus* was observed with each treatment. DMSO itself inhibited spore germination of *T. aurantiacus.*

4.3.7 *P. mosselii* improves cold stress potential of inoculated *japonica* rice plants in presence of the two phytopathogens

To evaluate the influence of *P. mosselii* on the survival of cold stressed rice plants that were treated with the two phytopathogenic fungi separately, LTSS was calculated on day 21 of recovery (Figure 4.7). Results showed that *japonica* plants inoculated with *P. mosselii* had better survival compared to uninoculated plants when challenged with *F. fujikuroi* or *T. aurantiacus* (Figure 4.7A), while *indica* plants inoculated with *P. mosselii* had lower survival compared to uninoculated plants when challenged with *F. fujikuroi* (Figure 4.7B). This suggests that factors such as rice genotype as well as bacterial and fungal genotypes influence plant-microbe interactions. While *P. mosselii* positively influenced cold survival of *japonica* plants in the presence of the two phytopathogenic fungi, it had a negative effect on *indica* plants in the presence of *F. fujikuroi*.

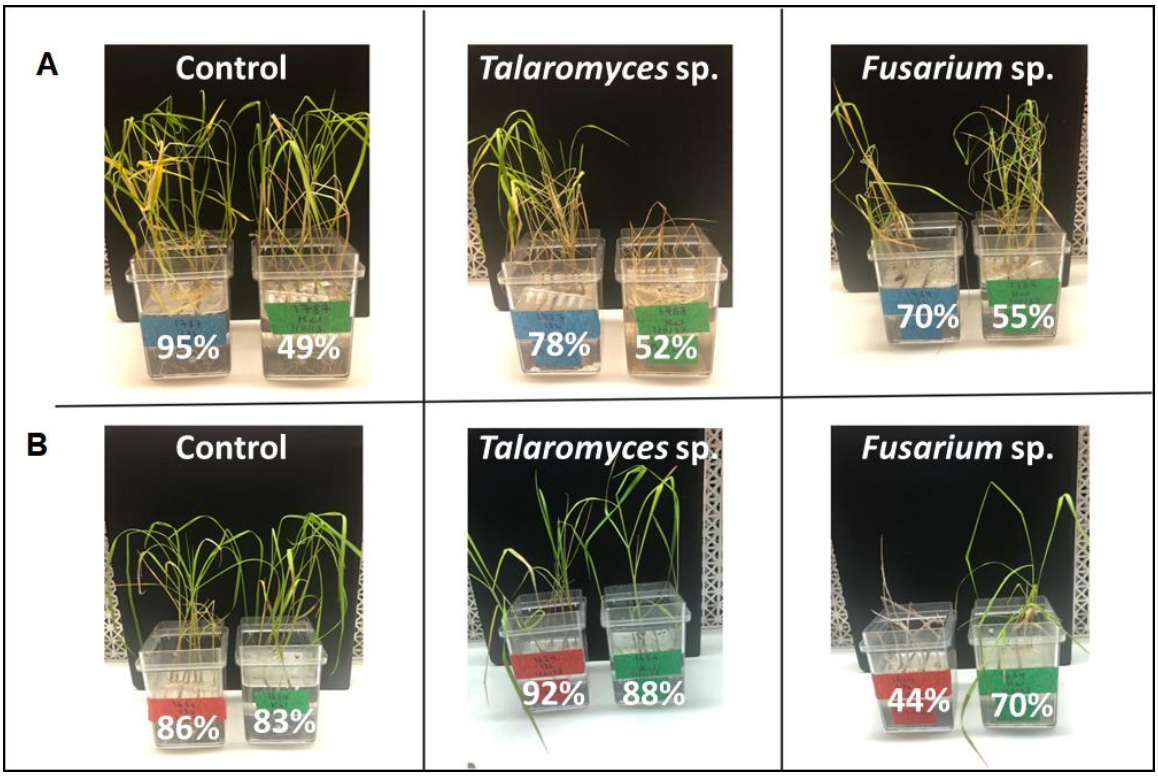

Figure 4.7 *In vivo* spore inoculation on day 7 of rice seedlings after *P. mosselii* rice seed inoculation. From left to right: control seedlings (without any phytopathogen inoculation); plants treated with *Talaromyces* sp.; plants treated with *Fusarium* sp. **A.** *Japonica* seedlings on day 21 of recovery. Boxes with blue labels contain plants inoculated with *P. mosselii* and boxes with green labels contain bacterial-free plants. **B.** *Indica* seedlings on day 21 of recovery. Boxes with red labels contain plants inoculated with *P. mosselii* and boxes with green labels contain bacterial-free plants. Statistical analyses: two-tailed student t-test: $p > 0.05$

4.4 Discussion

When plants are grown under stress conditions, active growth inhbition occurs, which is undesirable for crop productivity. This is one of the defense mechanisms that plants apply to adapt to stress conditions (Heng Zhang et al., 2020). Undesirable conditions cause the plants to use energy and resources toward stress response/survival and consequently, the process of growth and development and grain yield decreases. Furthermore, many genes and transcription factors become upregulated by stress sensing and signaling inside plants to protect them from the damage that can be caused by stress conditions (Yadav, 2009). In addition to a plant's internal responses, the plant microbiome plays an important role in stress defense mechanisms inside plants (Ngalimat et al., 2021). When plants are under stress, roots release specific compounds, known as root exudates, into the soil environment which facilitates beneficial interactions between plant roots and microbes. This also occurs under normal growth conditions, but the type of root exudates are different than under stress conditions (Chai & Schachtman, 2021).

It is known that *Pseudomonas* and *Bacillus* genera have the ability to fight against plant pathogens (Gutiérrez-Santa Ana et al., 2020; Khadiri et al., 2023). *Pseudomonas mosselii* is a bacterium known for its abilities in protecting and improving the growth of different plant species under various undesirable conditions including biotic and abiotic stress (Jha et al., 2009; Karkera et al., 2013; Lal et al., 2016; Lal et al., 2016; Alaa, 2018; Wei et al., 2018; Kumar & Meena, 2019; W. Li et al., 2020; Maghboli Balasjin et al., 2022; S. Li et al., 2023; Maghboli Balasjin et al., 2023, in revision). The bacterial whole genome assembly statistics identified this *Pseudomonas mosselii* isolate with 99.23% average nucleotide identity (ANI) threshold (Maghboli Balasjin et al., 2022). *P. mosselii* is a beneficial bacterium for growth and development of rice plants from the Krasnodarskij 3352 *japonica* variety as well as for their cold tolerance improvement and of plants from the Carolino 164 *indica* variety (Maghboli Balasjin et al., 2023, in revision; Maghboli Balasjin et al., 2022). It has gene clusters against phytopathogenic fungi (Wu et al., 2018) with different mechanisms that can protect plants ,such as production of insecticidal proteins (Wei et al., 2018), synthesis of the antimicrobial compounds such as xantholysin, production of antibiotic compounds such as pseudopyronines (Li et al., 2020), ACC deaminase synthesis (Maghboli Balasjin et al., 2022), upregulation of sugar content (Torre-ruiz et al., 2016), production of proteases, IAA and siderophore synthesis(Jha et al., 2009), pseudoiodinine biosynthesis (Li et al., 2023), exopolysaccharide production, and

therefore, biofilm formation (Alaa, 2018). In addition to these protective mechanisms, *P. mosselii* can protect plants through priming their defenses by increasing the amounts of glutathione and proline in *indica* (Carolino 164) and *japonica* (Krasnodarskij 3352) varietal rice plants, respectively (Maghboli Balasjin et al., 2023; in revisiond). Priming rice plants with glutathione and proline will prepare rice plants to better respond to cold temperature stress, because both compounds are known for detoxification of excess amounts of ROS (Maghboli Balasjin et al., 2023, in revision). When plants are under cold stress, ROS will damage plant cells through lipid and protein peroxidation (Hayat et al., 2012; Phan & Schläppi, 2021).

The increase in the amount of CO_2 in the atmosphere causes climate change and global warming as well as temperature fluctuations and therefore, has the potential to increase the reproduction of opportunistic phytopathogens (Sachin Gupta et al., 2018). Crop productivity decreased by 20-30% due to phytopathogen attacks and specifically rice productivity decreased by 37.4% (Sachin Gupta et al., 2018). Interestingly, results of my study showed that *P. mosselii* can protect Krasnodarskij 3352, *japonica* variety rice plants against fungal pathogens during the recovery period (after cold stress).

The two fungi that were isolated from *japonica* (Krasnodarskij 3352) and *indica* (Carolino 164) varieties in this study were identified as *Talaromyces aurantiacus* and *Fusarium fujikuroi*, respectively, based up ITS sequencing. *F. fujikuroi* is known for bakanae disease in rice plants (Chung et al., 2016). Bakanae disease causes morphological and color abnormalities such as dwarfed plant growth, yellowish-green leaves and a large angle between the leaf and stem in rice plants, which ultimately affects rice grain yield (Chung et al., 2016; Cheng et al., 2020). On the other hand, *T. aurantiacus* is known as a phosphate solubilizing fungus (Devi et al., 2020; Kaur et al., 2022) which was found in the rhizosphere of the medicinal plant *Ocimum tenuiflorum* "Tulsi" with antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (N. Rani et al., 2019), and in the rhizosphere of moso bamboo with phosphate solubilizing ability (Kaur et al., 2022). Therefore, this fungus seems to be a non-phytopathogen for those two plant species, but based on the observations in my study, the isolated strain of *T. aurantiacus* was phytopathogenic for *japonica* (Krasnodarskij 3352) varietal rice plants.

To understand the possible mechanism(s) of antifungal activites by *P. mosselii, in vitro* antifungal assays against spore germination and vegetative growth of *T. aurantiacus* and *F. fujikuroi* were performed. Results of *in vitro* antifungal activity against spore germination showed that live cells of *P. mosselii* had

negative effects on both *T. aurantiacus* and *F. fujikuroi*, while heat treated dead cells of *P. mosselii* only had negative effects on *T. aurantiacus*. Based on antiSMASH genome analysis (Blin et al., 2021) of *P. mosselii*, this bacterium can produce different secondary metabolites with antifungal activity such as pyoverdine, pseudopyronine A/B, pseudomonine, tolaasin I/F and fragin (Table 4.7) (Stintzi et al., 1996; Stintzi et al., 1999; Mercado-Blanco et al., 2001; Jo et al., 2011; Bauer et al., 2015; Jenul et al., 2018; Kang et al., 2018). One possibility is that there could be two or more different secondary metabolites that inhibit spore germination of the two phytopathogenic fungi. Some of these secondary metabolites could be heat-resistant.

On the other hand, live cells of *P. mosselii* inhibited vegetative (hyphal) growth of *F. fujikuroi*, while they had no significant effect on hyphal growth of *T. aurantiacus*. According to Rapid Annotations using Subsystems Technology (RAST) genomic analysis (Brettin et al., 2015), *P. mosselii* contains sequences for chitin and N-acetylglucosamine utilization. Specifically, the genes are annotated as N-acetylglucosamine-6-phosphate deacetylase (*NagA*), glucosamine-6-phosphate deaminase (*NagB*), predicted transcriptional regulator of N-acetylglucosamine utilization, GntR family (*NagR*) and the chitin binding protein (*Chinitoluytic_Enz*). Chitin is a polysaccharide and a primary component of fungal cell walls, as well as glucans (Lenardon et al. 2010; Matic et al. 2016; Min et al. 2020). However, according to studies by Abo Elsoud and El Kady (2019), not all fungi contain chitin in their cell walls. All the mentioned annotated genes in *P. mosselii* can utilize chitin of the fungal cell wall. Furthermore, the *NagA* gene in *P. mosselii* is also responsible for sialic acid metabolism, which is known for antifungal defenses (Dewi et al., 2021). Based on this information, it was assumed that *P. mosselii* can utilize chitin and N-acetylglucosamine of the *F. fujikuroi* cell wall and therefore inhibit vegetative growth of this pathogenic fungi (Figure 4.2B). Overall, *in vitro* antagonism showed that live cells of *P. mosselii* inhibited spore germination of both *T. aurantiacus* and *F. fujikuroi* (Figure 4.2A). However, the vegetative growth of *F. fujikuroi* was negatively influenced only by live cells of *P. mosselii* (Figure 4.2B). Dead cells of some bacteria release amino acids or volatile organic compounds (VOCs; Ebadzadsahrai et al., 2020; Smakman & Hall, 2022). Interestingly, dead cells of *P. mosselii* only inhibited spore germination of *T. aurantiacus* and had no effect on either spore germination or vegetative growth of *F. fujikuroi* (Figure 4.3). The

bacterial cell-free supernatant of *P. mosselii* had no effect on spore germination or vegetative growth of either phytopathogenic fungus (Figure 4.2C).

In vivo antagonism indicated that *P. mosselii* controlled *T. aurantiacus* pathogenicity in the *japonica* (Krasnodarskij 3352) variety of rice plants during the recovery period, while this bacterium had a negative effect on the *indica* (Carolino 164) variety when plants were challenged with *F. fujikuroi* (Figure 4.7). For this experiment, because plants grew in a sealed system (Figure 4.1) to avoid any outside contamination , the very humid area inside the closed magenta boxes might have caused the plants to survive better in comparison to open conditions (Maghboli Balasjin et al., 2023, in revision) where almost half of the uninoculated cold stressed plant population died on day 7 of recovery (34% and 55% of uninoculated *japonica* and *indica* survived, respectively). Therefore, LTSS was calculated on day 21 during the recovery period.

It was hypothesized that there were four scenarios for how *P. mosselii* controlled these two phytopathogenic fungi. Based on *in vivo* results, one scenario was that the interactions between the microorganisms and rice plants were dependent on the genotype of each of these microorganisms and the plants. This is compatible with previous results (Maghboli Balasjin et al., 2023, in revision; Maghboli Balasjin et al., 2022) that indicated that not all PGPB behave similarly in one specific rice variety. When *P. mosselii* interacted with *F. fujikuroi* inside *indica* (Carolino 164) variety plants, the consequences were different from when these two microorganisms were inside the *japonica* (Krasnodarskij 3352) variety rice plants. Based on *in vitro* results related to spore germination of fungi, the second scenario was that *P. mosselii* produced two or more different secondary metabolites while some were heat-resistant and had specific genotype dependent interactions with the two different pathogenic fungi. Pseudomonine, Crochelin A and Pyoverdin are siderophores and have the ability of inhibiting fungal growth (Stintzi et al., 1996; Stintzi et al., 1999; Mercado-Blanco et al., 2001; Maindad et al., 2014; Kang et al., 2018; Baars et al., 2018). Pseudopyronine A and B, Tolaasin I and F, Lankacidin C are known as antibiotics with strong antimicrobial activities (Hiratsu et al., 2000; Suwa et al., 2000; Mochizuki et al., 2003; Jo et al., 2011; Bauer et al., 2015). Pseudopyronine A and B disrupt cell membranes and inhibit fatty acid synthase (Bauer et al., 2015). Tolaasin I and F destroy cell structure by forming pores on cellular membrane (Jo et al., 2011). Lankacidin C is mostly inhibiting Gram-positive bacterial growth by disrupting bacterial ribosomes

(Hiratsu et al., 2000; Suwa et al., 2000; Mochizuki et al., 2003). From the *in vitro* results related to the vegetative growth of fungi, the third scenario was that *P. mosselii* contained specific genes targeting cell walls of *F. fujikuroi* and therefore inhibited further hyphal growth of this fungus. Finally, due to the negative effect of unfiltered live and dead cells of *P. mosselii* on two phytopathogens, the fourth scenario is that *P. mosselii* contains cell membrane components with antimicrobial properties.

Further investigation needs to be done to understand the interactions between *P. mosselii* and the two phytopathogens in the different rice varieties. Studies using High-performance liquid chromatography (HPLC) and Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) are powerful analytical techniques with highly specified analysis capabilities which can determine which of the mentioned secondary metabolites of *P. mosselii* were involved in controlling *T. aurantiacus* and *F. fujikuroi*. In addition, more studies should be done to understand whether live cells of *P. mosselii* produce inhibitory metabolites only in the presence of these phytopathogens (simultaneous bacteria-fungi interactions). In addition, because dead cells of *P. mosselii* also had a negative effect on spore germination of *T. aurantiacus*, more *in vitro* experiments should be performed with dead cells of this bacterium to understand whether this antagonism is dependent on the cell membrane of *P. mosselii*.

This study opens a novel view on plant interactions with PGPB while two different biotic and abiotic stresses are involved. While the interactions between PGPB and plants are genotype dependent, the reactions of beneficial and pathogenic microorganisms could harm or help a specific plant genotype.

Chapter 5: Overexpression of *OsMADS27* improves cold tolerance in Asian rice (*Oryza sativa* L.)

Abstract

OsMADS27 belongs to the MADS-box family of transcription factors (TFs) and is known to improve salinity tolerance when overexpressed in transgenic *Oryza sativa*. *OsMADS27* is upregulated during cold stress in wild type rice plants. In this study, effects of overexpressing and knocking out *OsMADS27* in two different *indica* and *japonica* rice varieties on cold tolerance were investigated. Preliminary results showed that when *OsMADS27* was overexpressed in rice plants, the survivability of cold stressed plants increased. Also, primary root growth was inhibited when *OsMADS27* was overexpressed in the *japonica* rice variety. RNA sequencing results showed an increase in transcriptomes of defense and peroxidase genes. Taken together, preliminary results of this study suggest that *OsMADS27* is crucial for root growth regulation and response to abiotic stresses by upregulating defense and peroxidase genes.

Keywords: *OsMADS27*, *Oryza sativa*, Primary root, Cold tolerance

5.1 Introduction

Recently, genetic manipulation of plants has become common due to its benefits for modern agriculture. For basic research, it facilitates the study of biological processes and helps scientists to produce specific types of plants that are resistant to undesirable environmental conditions (Rogers & Parkes, 1995). Along with its benefits, there are still some challenges including expressing transgenes and induction of embryogenic callus for producing suitable transgenic plants (Gordon-kamm et al., 2019).

Rice (*Oryza sativa* L.) is a monocot model crop for studying biotic and abiotic stresses because of its adaptability to different environments (Romero & Gatica-Arias, 2019; Cockerton et al., 2020) and its genome has been sequenced (Panigrahy et al., 2009). Due to population increase from 7 billion to 9.2 billion in 2050, the food supply needs to be increased as well. As rice is the staple food for half of the world's population, to keep up its production to meet the world's demand, different approaches such as the use of biofertilizers and high-throughput rice transformation have become the primary targets (Duan et al., 2012). Recently, global warming has become a major concern, because one of its consequences is extreme fluctuations between warm and cold weather conditions (Johnson et al., 2018), having serious consequences on crop production.

Rice plants are particularly sensitive to low temperature exposure during the early stages of seedling development. *O. sativa* has two subspecies, *indica* and *japonica*, of which *indica* is generally more sensitive to low temperature exposure compared to *japonica* (Liu, et al., 2018). Cold tolerance in rice is a quantitative trait and involves simultaneous regulation, at different levels, of various genes and gene

families. One gene family with considerable research interest is the MADS-box family (Castelán-Muñoz et al., 2019).

MADS-box transcription factors, encoded by MADS-box genes, are involved in floral organ specification and have also been implicated in several processes of plant growth and development (Castelán-Muñoz et al., 2019). Recent studies have explored genomic localization, protein motif structure, phylogenetic relationships, gene structure, and expression of the entire MADS-box family in model plants such as rice and *Arabidopsis thaliana* (Chen et al., 2016). Although a large amount of expression data based on transcriptomic analysis techniques is available in public databases, most functional studies of the entire MADS-box family have so far focused mainly on their roles in flowering time and flower development and not during young vegetative stages where plants are more susceptible to abiotic stresses. However, a few MADS-box genes have been shown to be involved in abiotic stress response mechanisms. For example, *OsMADS7* and *OsMADS87* are involved in plant sensitivity to heat stress (Chen et al., 2016; Zhang et al., 2018). Those two genes are thus negative regulators of abiotic stress tolerance.

In a search for positive regulators, *OsMADS27* has been previously filtered out as a promising candidate gene for regulating tolerance against abiotic stresses such as high salinity and potentially cold temperatures. For salinity stress, it was shown that compared to other MADS box genes, *OsMADS27* had a more crucial role in regulating high salinity tolerance than in regulating flowering time (Chen et al., 2018). For cold temperature stress, our lab showed that *OsMADS27* contained the peak single nucleotide polymorphism (SNP) for the quantitative trait locus (QTL) *qL4.2-6*, which was identified through genome-wide association study (GWAS) mapping (Shimoyama et al. 2020; and unpublished). Based on the observation that *OsMADS27* mediated modulation of salt tolerance is nitrate dependent (Chen et al., 2018) and because some root associated plant growth pomoting bacteria (PGPB) can help with nitrate uptake, functional studies using transgenic overexpression and knockout lines were performed to determine whether *OsMADS27* also plays a positive role in regulating cold temperature stress tolerance in rice. A positive result would make it worthwile to test whether *OsMADS27* overexpression and application of PGPB have a synergistic effect on enhancing cold tolerance potential. Furthermore, having *OsMADS27* overexpressed and applying PGPB, will be environmentally beneficial due to being an efficient replacement for nitrogen-fertilizers. Expression of *OsMADS27* is induced by nitrate (Alfatih et al., 2022)

and PGPB are able to provide nitrate to plants through nitrogen fixation. Hence, it is expected to identify positive interactions between *OsMADS27* and some specific nitrogen-fixing PGPB.

Recent genome editing techniques helped facilitate the production of genetically engineered crops. The most efficient genome editing techniques is based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes (CRISPR/Cas; Romero and Gatica-Arias 2019). CRISPR/Cas9 (derived from *Streptococcus pyogenes*) is a suitable system for knocking out rice genes and can be used to produce transgenic rice plants (Jiang et al., 2019). In addition to the CRISPR/Cas method, *Agrobacterium*-mediated transformation of rice plants is another critical method which was established in 1994 for rice as the first monocotyledonous system previously thought to be recalcitrant to *Agrobacterium* (Duan et al., 2012). In this study, we used both the CRISPR/Cas and *Agrobacterium*-mediated transformation methods to knock out and overexpress the *OsMADS27* transcription factor gene in rice to investigate the response of *OsMADS27* to cold temperatures.

To the best of our knowledge, the expression profiles of *OsMADS27* in the cold and potential impact on regulating low temperature stress tolerance in young seedlings have not yet been investigated. When plants are under stress, they are more susceptible to diseases and, therefore, chemical fertilizers containing nutrients will be applied to boost their immunity against pathogens. Creating transgenic plants overexpressing *OsMADS27* that increase rice cold tolerance potential compared to wild type plants will reduce the application of chemical fertilizers and is expected to have a positive effect on the environment.

5.2 Material and Methods

5.2.1 Vector construction for overexpressing *OsMADS27*

To generate the overexpression construct, *Bam*HI containing forward primer (site underlined), GGATCCTGACCGGCCTGGAGCTAGC, and *EcoR*I containing reverse primer (site underlined), GAATTCGCTCCAGGCCGGTCATG, were used to amplify the full length cDNA of *OsMADS27*. Amplified cDNA was cloned into the *Bam*HI-*EcoR*I sites of the pPZP211 binary vector containing the strong MAC promoter and mannopine synthase (mas) terminator for generating overexpression lines (Shi et al., 2020). *Agrobacterium tumefaciens* strain EHA105, which is ideal for rice transformation via T-DNA transfer, was used to generate transgenic rice plants following the methods of Sahoo et al. (2011).

5.2.2 gRNA design and gRNA-Cas9 plasmid construction for knocking out *OsMADS27*

The coding sequence for *OsMADS27* was obtained from the rice genome browser website (http://rice.plantbiology.msu.edu/). To design a guide RNA (gRNA) spacer sequence located next to the Cas9 specific protospacer adjacent motif (PAM) NGG, the CRISPR-PLANT v2 data set of for University of Arizona (https://www.genome.arizona.edu/crispr2/instruction.html) was used. The website used to screen for the potential "locations" for knocking out a gene in rice was http://omap.org/crispr/.

Note: The gRNA spacer sequence should be specific with a rank between 0.0 and 1.0 in the CRISPR-PLANT database to avoid off-target mutations. It is ideal to find spacer sequences which are specified to restriction nuclease enzymes for genotyping T_0 plants in order to detect transgenic plants. Two adaptor sequences including 5'-GGCA-3' and 5'-AAAC-3' for forward and reverse oligos, respectively, were added for cloning.

Sequences of paired DNA oligos (without cloning adaptors) to construct gRNA were as follows:

- Forward: 5'-AAGTCAGTTATAGATCGGTA-3'
- Reverse: 5'-TACCGATCTATAACTGACTT-3'

The final oligo sequences were as follows:

- Forward: 5'-GGCAAGTCAGTTATAGATCGGTA-3'
- Reverse: 5'-AAACTACCGATCTATAACTGACTT-3'

For gRNA-Cas9 plasmid construction, the binary vector pRGEB31 (Addgene, Watertown, MA) was used to generate knockout lines. This vector confers Kanamycin resistance in bacteria and was maintained in *Escherichia coli* (*E. coli*) strain DH5a. Construction of the gRNA-Cas9 plasmid was done as previously described by Xie et al. (2014a) with minor modifications. Briefly, pRGEB31 was cut with BsaI (NEB, Ipswich, MA) generating 5'-TGCC-3' and 5'-GTTT-3' overhangs and purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD). To check whether the pRGEB31 plasmid was fully digested, 5% of the restriction enzyme digestion reaction was transformed into competent DH5a cells and spread onto LB agar medium containing 50 µg/mL Kanamycin. No growth on LB agar medium containing the Kanamycin antibiotic was expected if the plasmid was fully digested.

Next, DNA oligo-duplexes were prepared by mixing forward and reverse oligos (1 µl each), 1 µl 10X T4 DNA ligase buffer, and 7 µl sterile distilled water. The mix was incubated at 37˚C for 1 h, 95˚C for 10 min, then cooled down to 25˚C. DNA concentration was measured with the Nanodrop spectrophotometer (ng/µl). The oligo-duplex was diluted 1:200 and ligated into the BsaI digested pRGEB31 vector (2 µl BsaI digested vector, 1 µl diluted oligo-duplex, and 0.5 µl sterile distilled water). Tubes were incubated at 65˚C for 15 min and immediately transferred on ice (30 s) and 0.5 µl 10X T4 DNA ligase buffer along with 1 µl T4 ligase (NEB) were added to each tube. Tubes were incubated overnight at room temperature in the dark. The same steps were repeated to prepare a control tube without addition of the diluted oligo-duplex.

After overnight incubation, transformation was done by incubating the ligated tubes at 65˚C for 15 min. Then, 2 µl of ligated pRGEB31 was added to 50µl of competent DH5a cells on ice. Tubes were incubated on ice for 30 min followed by incubating briefly at 42˚C for 45 s and transferring on ice for 1 min. 450 µl LB liquid medium was added to each tube at room temperature and tubes were incubated at 37˚C for 2 h. 500 µl of the construct was spread onto LB agar medium containing 50 µg/mL Kanamycin antibiotic and plates were incubated at 37˚C overnight. Due to ligation of the gRNA spacer sequence containing compatible BsaI ends into the pRGEB31 plasmid at this step, growth on LB agar medium containing Kanamycin antibiotic was expected.

Colonies from overnight LB plates, which contained the pRGEB31 plasmid, were transferred to LB liquid medium and incubated at 37˚C overnight on a shaker. Then, plasmids were extracted using the QIAGEN Plasmid mini-prep kit and sent for sequencing to the Genewiz sequencing company (www.genewiz.com) in order to identify plasmids carrying the correct construct.

5.2.3 Introducing *OsMADS27* overexpressing and knocking constructs into rice callus

Following established rice transformation methods (Silva et al., 2001; Sahoo et al. 2011; Xie et al., 2014; Shan et al., 2014; Luu et al., 2020)**,** three steps were done to create transgenic rice plants. First, binary vectors pPZP211::MAC-*OsMADS27*-mas and pRGEB31::*OsMADS27*-gRNA were introduced into *Agrobacterium* strain EHA105 using the freeze-thaw method. Competent frozen *Agrobacterium tumefaciens* cells were thawed on ice and 100-200 ng of the selected plasmid was added to the bacteria and incubated on ice for 5 min. The whole mixture was submerged in liquid nitrogen for 5 min and transferred

to a 37˚C water bath for another 5 min. 1 mL of LB was added to the mixture and tubes were incubated at 30˚C on a shaker (200 rpm) for 4 h. Then, tubes were centrifuged (1,073 x g) for 5 min and the supernatant discarded and replaced with 100 µL LB. The liquid in tubes were spread on LB agar medium containing Kanamycin (50 µg/mL) to select for pRGEB31 and spectinomycin (50 µg/mL) and streptomycin (50 µg/mL) to select for pPZP211 and incubated at 30˚C for 2-3 d. Finally, *Agrobacterium* colonies carrying the construct of interest were selected and grown in YEP broth for 48 h at 30˚C and glycerol stocks (15%) were made and stored at -80˚C.

Second, *Agrobacterium* carrying the overexpression (pPZP211) or knockout (pRGEB31) constructs were added to rice callus to allow the bacteria to transfer T-DNA into the rice genome. Seeds of Krasnodarskij 3352 and Kasalath representing *japonica* and *indica* subspecies, respectively, were dehulled and surface sterilized following these steps: shake seeds in ethanol (70%) for 1 min, rinse with sterile ddH_2O three time (1 min per rinse), shake with sodium hypochlorite (50%) (15 min), and rinse with sterile ddH_2O three time (1 min per rinse). Surface sterilized seeds were dried on sterile filter paper in a sterile hood and transferred to N6D (callus initiation) medium (up to 15 seeds in each plate) and incubated for 1 month under 12/12 hours light/dark cycles at room temperature. Callus emerging from the coleoptiles of each seed was cut and transferred to another N6D medium (up to 15 calli in each plate) and incubated for 3 days under the same condition as mentioned. *Agrobacteria* carrying knockout and overexpression constructs were grown in YEP liquid medium containing Kanamycin (50 µg/mL) to select for knockout and spectinomycin (50 µg/mL) and streptomycin (50 µg/mL) to select for overexpression vectors and incubated on a shaker (200 rpm) at 30˚C overnight. 20 mL of infection medium was added to a beaker and 2 drops of YEP broth containing *Agrobacterium* carrying the interested constructs were added and shaken for 4 h at room temperature. Calli were removed from the N6D medium and added to the beaker and shaken for 5 min at room temperature to allow attachment of *Agrobacteria* carrying the interested constructs to rice calli. The inoculated calli were put on sterile filter papers in autoclaved glass plates and dried in a sterile hood for 45 min to 1 h and then incubated at 25˚C in the dark for 3 d. Calli were transferred to selection medium containing Hygromycin B (50 µg/mL), Carbenicillin (100 µg/mL) and Vancomycin (100 µg/ml) to select for knockout and Carbenicillin (100 µg/mL) along with G418 (100 µg/mL) for overexpression constructs and incubated for 1 month under 12/12 hours light/dark cycle at

room temperature. Cali were transferred to selective medium two more times. Only white and alive calli were selected and transferred to regeneration medium I containing the same antibiotics for knockout and overexpress constructs (each plate was filled with 5 calli). Plates were incubated for 1 to 2 months under 12/12 hours light/dark cycles at room temperature until green callus with shoots and roots appeared. The green calli were transferred to regeneration medium II without any antibiotics and incubated for 3 weeks under 12/12 hours light/dark cycles at room temperature until plantlets with appropriate amounts of roots had regenerated, which were transferred to soil as T_0 plants.

Media composition for rice transformation are including (1) N6D (callus initiation) medium containing N6 salts (4 g/L), 2,4-D (2 mg/L), Casamino acids (300 mg/L), L-Proline (2.8 g/L), Sucrose (30 g/L), Gelrite (4 g/L), pH: 5.8, N6 vitamin stock solution (1mL added after autoclaving); (2) Liquid *Agrobacterium* infection medium containing N6 salts (4 g/L), 2,4-D (1.5 mg/L), N6 vitamin stock solution (1 ml/L), L-Proline (0.7 g/L), sucrose (68.4 g/L), glucose (36 g/L), pH: 5.2, the medium should be filter sterilized through 0.2 µm membranes and stored at 4˚C; (3) selection medium containing N6 salts (4 g/L), 2,4-D (2 mg/L), casamino acids (300 mg/L), L-Proline (2.8 g/L), sucrose (30 g/L), gelrite (4 g/L), pH: 5.8, after autoclaving 1 mL N6 vitamin stock solution, 1 mL Hygromycin B (50 mg/mL stock), 1 mL Vancomycin (100 mg/mL stock) and 1 mL Carbenicillin (100 mg/mL stock) should be added to the medium; (4) regeneration I medium containing MS salt + vitamin (4.43 g/L), kinetin (2 mg/L), casamino acids (2 g/L), sorbitol (30 g/L), sucrose (30 g/L), agar (8 g/L), pH: 5.8, after autoclaving 0.02 mg/L NAA, 1 mL Hygromycin B (50 mg/mL stock), 1 mL Vancomycin (100 mg/mL stock), and 2.5 mL Carbenicillin (100 mg/mL stock) should be added to the medium; (5) regeneration II medium containing MS salt + vitamin (4.43 g/L), myoinositol (100 mg/L), sucrose (30 g/L), gelrite (3 g/L), pH: 5.8.

Note: Antibiotic effects:

- Hygromycin selects transgenic plants
- Carbenicillin suppresses *Agrobacterium* sp. (Gram-negative) growth
- Vancomycin suppresses some Gram-positive bacteria growth
- G418 blocks polypeptide synthesis by inhibiting the elongation step in prokaryotic cells

Third, transgenic plants were validated using PCR. DNA was extracted from leaves of T_0 plants using 180 µL of DNA extraction buffer and smashing leaves with clean plastic pestles. Extracts were stored at 4˚C overnight to make sure the DNA extraction process was completely done.

DNA extraction buffer was prepared following the method of Edwards et al. (1991) with minor modifications. 200 mL Edwards solution was prepared as below:

- Ethylenediamine tetraacetic acid (EDTA): 25 mM
- Tris-HCl (pH = 7.5): 200 mM
- NaCl: 250 mM
- Sodium dodecyl sulfate (SDS): 0.5%

500 mL Tris-EDTA (TE) buffer was prepared as below:

- Tris-HCl (pH = 8): 10 mM
- EDTA: 1 mM

Edwards solution was diluted to 10-fold with TE buffer.

This method was used for DNA extraction of *OsMADS27* overexpressing transgenic plants. For knockout transgenic plants, the cetyltrimethylammonium bromide (CTAB) extraction method of Doyle and Doyle (1987) was followed.

For validating overexpressing transgenic plants, DNA extracted from different plants was used to perform PCR with two pairs of primers: one was specific for the *OsMADS27* overexpression construct (*Mas1*, amplifying the mannopine synthase terminator) and one was a control for genomic rice DNA (*Ubq2*, amplifying an endogenous *ubiquitin* gene). Through gel electrophoresis analyses of PCR products, *OsMADS27* overexpressing transgenic and segregating wild type plants were identified. Homozygous or heterozygous lines could not be recognized at this stage. Through genotyping the progenies of T_0 plants, homozygous or heterozygous transgenic plants were identified. To validate knockout transgenic plants, DNA from different plants was used to perform PCR with three pairs of primers: two were specific for the *OsMADS27* knockout construct (*Nos* and *Cas9*, amplifying gRNA linked genes neomycin phosphotransferase II and CRISPR associated gene 9, respectively) and one was a control for genomic rice DNA (*Ubq5*, amplifying an endogenous *ubiquitin* gene). Primer sequences are listed in Table 5.1.

Table 5.1 Primer sequences designed for detecting transgenic or segregating wild type plants.

Primer	Sequence
Mas1 Forward	CCATTTGGGCTGAATTGAAGAC
Mas1 Reverse	CAGATAAAGCCACGCACATTTAG
Ubq2 Forward	AATCAGCCAGTTTGGTGGAG
Ubq2 Reverse	ACTGCTGTCCCACAGGAAAC
Nos Forward	TCGTTCAAACATTTGGCAAT
Nos Reverse	CCTAGTTTGCGCGCTATATTT
Cas9 Forward	CAGCCAGGAAGAGTTCTACAAG
Cas9 Reverse	CATTCCCTCGGTCACGTATTT
Ubq5 Forward	ACCACTTCGACCGCCACTACT
Ubq5 Reverse	ACGCCTAAGCCTGCTGGTT

PCR products to analyze the extent of genomic editing done by the *OsMADS27* gRNA were ligated into the pGEM-T vector (Promega, Madison, WI) and transformed into competent DH5a cells and used for sequencing. To amplify the gRNA targeted region of *OsMADS27*, primers F2: GGAGGT-GTGTTAGATGGATG and R2: TGGTGTTCTACTGTGTCTATATG were used, and PCR products purified with the QIAquick PCR purification kit. The concentration of DNA was measured using the Nanodrop system.

Note: To calculation the correct amount of DNA for the ligation reaction, the online neb calculator (https://nebiocalculator.neb.com/#!/ligation) was consulted.

Plasmid were extracted from individual DH5a colonies selected on Ampicillin (100 µg/mL) using the QIAGEN Plasmid Mini kit and glycerol stocks (20%) were prepared from leftover cells and stored at -80˚C for further usage. DNA concentrations of extracted plasmids were measured using the nanodrop system and based on the readings, plasmid DNA samples were diluted to a 100 ng/µL concentration. Plasmids were digested with SalI to identify plasmids containing inserts. Plasmid with inserts were sent to Genewiz (www.genewiz.com) for sequencing to analyze the type of mutation in each sample. Knockout sequences were alignment with wild type sequences and analyzed using the https://web.expasy.org/sim/ site.

5.2.4 Evaluating growth and cold tolerance of transgenic rice plants (T_1 plants)

Progenies of T_0 plants (named T_1 plants) and wild type seeds were germinated in ddH_2O mixed with 0.1% sodium hypochlorite in the dark at 37˚C for 48 h. Germinated seeds were transferred to boxes filled with ddH_2O and placed in a growth chamber (12h/12h 28˚C/25˚C light/dark cycles). When plants were 14 days old, cold stress was applied (2d10˚C for Kasalath and 4d4˚C for Krasnodarskij 3352

representing *indica* and *japonica*, respectively) in 12h/12h light/dark cycles. After cold stress, plants were placed back in the 28˚C/25˚C growth chamber for a 7-day recovery period and % low temperature seedling survivability (LTSS) was calculated [(# green and healthy-looking plants/# total plants) x100]. Root and shoot growth of each plant was measured when plants were 8, 9, 10, 11, 12, 13, 14 and 22 days old.

5.2.5 RNA sequencing (RNAeq) of transgenic and wild type rice plants

Transgenic progenies of T_1 plants that were genotyped as *OsMADS27* overexpressing and knockout plants and wild type rice plants of both subspecies were germinated and grown in a growth chamber until the flowering stage. Progenies of T_1 plants (named T_2 plants) were genotyped and grown until the flowering stage. Progenies of T_2 plants (named T_3 plants) were germinated and genotyped and overexpressing and knockout plants were transferred to a cold chamber along with wild type plants. Cold stress temperatures and stress duration were the same as previously mentioned (2d10˚C for Kasalath and 4d4˚C for Krasnodarskij 3352 representing *indica* and *japonica*, respectively). Control plants remained in the growth chamber in warm conditions until day 14. Cold stressed plants were collected right after cold stress and non-cold stressed plants were also collected on day 14 for RNA extraction. Plant tissues were ground to a fine powder under liquid nitrogen and stored at -80˚C until RNA extraction. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). RNA samples were sent to the Novogene biotechnology company (https://en.novogene.com) in Sacramento, CA, to analyze the rice transcriptome and to identify potential *OsMADS27* targets and other genes influenced by *OsMADS27*.

5.2.6 RNAseq analysis validation

Based on RNAseq data from *OsMADS27* overexpressing plants, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was done using specific primers related to three peroxidase genes including *OsPRX25*, *OsPRX91*, and *OsPRX92*. To confirm increased *OSMADS27* mRNA levels in transgenic plants, qRT-PCR was done. Primers are listed in Table 5.2.

Table 5.2 Primer sequences designed for measuring peroxidase and *OsMADS27* transcripts in transgenic plants.

Primer	Sequence
OsPRX25 Forward	TTCGACAACGGCTACTT
OsPRX25 Reverse	ATCTTCACCATCGCCTT
OsPRX91 Forward 1	CTCCCAGACTGACATGAT
OsPRX91 Reverse 1	GGAACGCGAAGTTGTTG
OsPRX92 Forward 2	CAGCTGAAGCAGAACTACTA

OsPRX92 Reverse 2	CCGTTCGGGTTGATGAT
OsMADS27 Forward 11	GACCACGTCTTGATTGATG
OsMADS27 Reverse 11	GCTTAGGAGCAGTTGATTG
*Act1 F*orward 2	TCCTGATGGACAGGTTATC
Act1 Reverse 2	CTTCATGATGGAGTTGTATGT
18S Forward	CTACGTCCCTGCCCTTTGTACA
18S Reverse	ACACTTCACCGGACCATTCAA

5.2.7 Evaluating growth parameters and cold tolerance of transgenic rice plants (T_4 plants)

After genotyping T_3 seedlings and isolating RNA from transgenic plants, extra transgenic seedlings were grown to maturity and progenies of T_3 plants (named T_4 seeds), overexpressing and knockout Kasalath and overexpressing Krasnodarskij 3352, were grown for 14 d in a growth chamber (28˚C/25˚C 12h/12h light/dark cycles). Growth of shoots and roots was measured on day 14 and Kasalath and Krasnodarskij 3352 seedlings were then transferred to cold chambers with 10˚C (2d) and 4˚C (4d), respectively, under 12 h/12 h light/dark cycles. Right after cold stress, electrolyte leakage (EL) of transgenic and wild type leaves was measured following the methods of Shi et al. (2020). Finally, plants were placed back to the growth chamber for a 7-day recovery period at 28˚C/25˚C and 12 h/12 h light/dark cycles.

Note: Genotyping of T_4 plants was done using below primers (Table 5.3):

Table 5.3 Primer sequences designed for detecting transgenic plants and wild type plants.

Primer	Sequence
Mas1 Forward	CCATTTGGGCTGAATTGAAGAC
Mas1 Reverse	CAGATAAAGCCACGCACATTTAG
OsGH3 Forward 1	GGGCTAATCATCCCACTGATAC
OsGH3 Reverse 1	CCCTGTTCACCTTCTCTTCATC
OsMADS27 Forward 11	GACCACGTCTTGATTGATG
OsMADS27 Reverse 11	GCTTAGGAGCAGTTGATTG

5.2.8 Evaluating growth parameters, cold tolerance, and salinity of transgenic rice plants (T_5 plants) with and without *P. mosselii*

Progenies of T_4 plants (named T_5 seeds) were used for bacterial inoculation and evaluating the influence of plant growth promoting bacteria on *OsMADS27* overexpressing plants (microbe-plant gene interactions). Following the methods of Maghboli Balasjin et al. (2022), T_5 seeds were inoculated with *P. mosselii*. Briefly, seeds were dehulled and surface sterilized 1 min in ethanol (70% vol/vol in dH_2O), rinsed

3x in sterile dH_2O, then soaked for 5 min in sodium hypochlorite (50% vol/vol in dH_2O) and rinsed 3x in sterile dH_2O. Half of surface sterilized seeds were soaked in a 10^8 cell mL^{-1} bacterial suspension and the other half was soaked in KCl solution without any bacteria (control seeds) on a shaker at room temperature (200 rpm) overnight. Then seeds were dried on sterile filter papers and germinated on Murashige-Skoog (MS) agar medium at 30˚C for 48 h. Germinated seeds were transferred to boxes and grown hydroponically in a 28˚C/25˚C growth chamber under 12 h/12 h light/dark cycles. Shoots and roots measurement were done with 14-day-old seedlings. Half of the seedling population was transferred to cold chambers (2d10˚C and 4d4˚C for Kasalath and Krasnodarskij 3352, respectively) and LTSS was calculated after a 7-day recovery period. The other half was treated with NaCl to apply salinity stress. Based on preliminary results, a 7-day exposure to NaCl at concentrations of 150 mM, 160 mM and 170 mM of 14-day-old wild type (WT) Kasalath and Krasnodarskij 3352 had different effects on plant survival (Table 5.4). The appropriate salt concentration to affect approx. 50% death (LT50) in WT Kasalath and Krasnodarskij 3352 plants was 155 mM NaCl. For T_4 plants, 150 mM NaCl was used, and concentrations of 150 mM and 155 mM were used for T_5 Kasalath and Krasnodarskij 3352, respectively. In each experiment, 14-day-old seedlings were transferred to the mentioned NaCl concentrations for 7 days and evaluation of salinity stress survival was done after a 7-day recovery period.

Table 5.4 Effect of different NaCl concentrations on survival of WT rice plants after a 7-day recovery period.

NaCl concentrations	Kasalath survival rate (%)	Krasnodarskij 3352 survival rate (%)
150 mM	50	100
160 mM	14	33
170 mM	0	42

5.3 Results

5.3.1 Genotyping validation of transgenic plants

To validate that T_0 rice plants were transgenic, PCR based genotyping of Kasalath and Krasnodarskij 3352 T_0 plants was performed. Gel electrophoresis was performed to identify the expected sizes of PCR products generated with primers specific for the overexpression construct (103 bp for *Mas1*) and for an endogenous *ubiquitin* gene as a positive control for the presence of rice genomic DNA (158 bp for *Ubq2*). Transgenic plants had both a *Mas1* and *Ubq2* band while segregating WT plants only had a

Ubq2 band (Figure 5.1). The pPZP211 plasmid and WT genomic DNA were used as positive and negative controls, respectively. All samples named T1_20 were shown to be transgenic plants and used for RNAseq analysis.

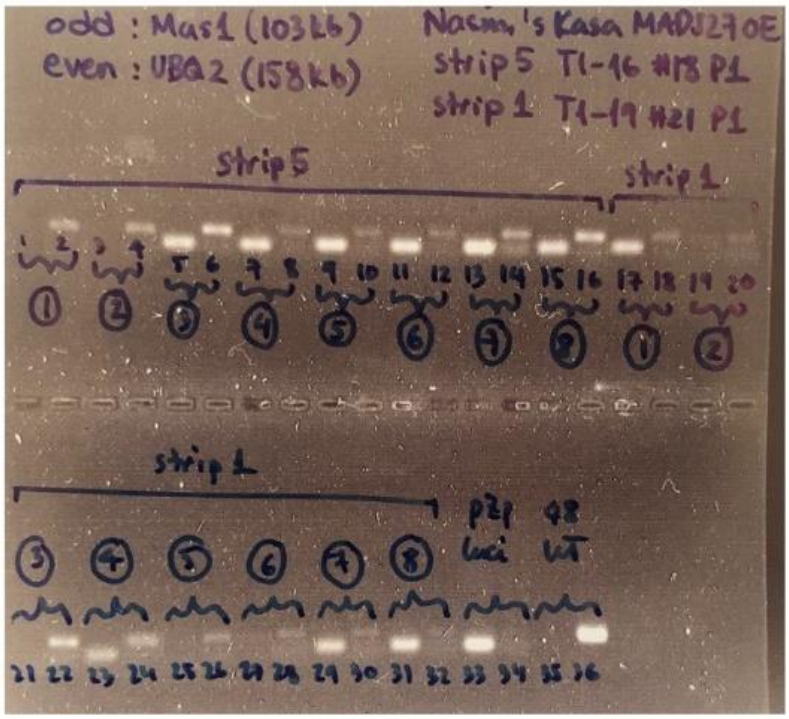

Figure 5.1 Gel electrophoresis of PCR products generated with *Mas1* (odd numbers) and *Ubq2* (even numbers) primers to select overexpressing transgenic and segregating WT plants. WT plants only had an *Ubq2* band (158 bp), while *OsMADS27* overexpressing plants had *Mas1* and *Ubq2* bands. This gel picture is representative for Kasalath plants. Eight Kasalath plants were genotyped and because there were two primers to test, two PCR products were generated for each plant. Positive control: PZP plasmid; negative control: WT rice plant.

For knockout (KO) plants, T_0 plants containing KO construct positive DNA (Figure 5.2) were used for PCR amplification of the *OsMADS27* gRNA target region, ligated into the cloning vector pGEM-T, and after digestion with the SalI restriction enzyme to confirm the presence of inserts (Figure 5.3), plasmid samples were sent for sequencing.

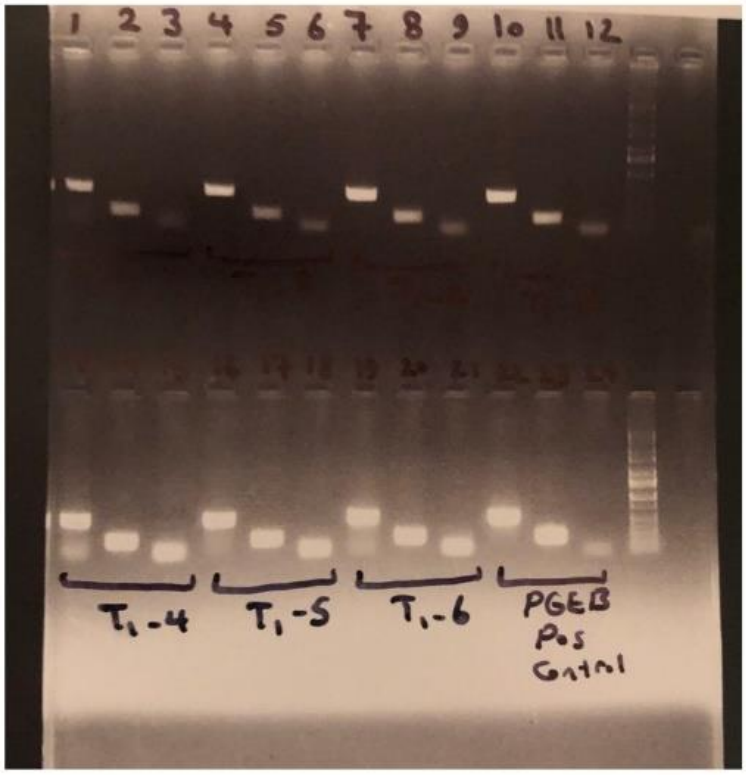

Figure 5.2 Gel electrophoresis of *Cas9*, *Nos*, and *Ubq5* specific PCR products to identify knockout construct containing transgenic plants. WT plants only had the *Ubq5* band, while transgenic plants had Cas9, Nos, and *Ubq5* bands. Samples 1, 4, 7, 10, 13, 16, 19 and 22 had *Cas9* bands, samples 2, 5, 8, 11, 14, 17, 20, 23 had *Nos* bands, and samples 3, 6, 9, 12, 15, 18, 21, 24 had *Ubq5* bands. This gel picture is representative for Kasalath plants. Seven Kasalath plants were genotyped and because there were three primers to test, three PCR products were generated for each plant. Positive control: PGEB plasmid.

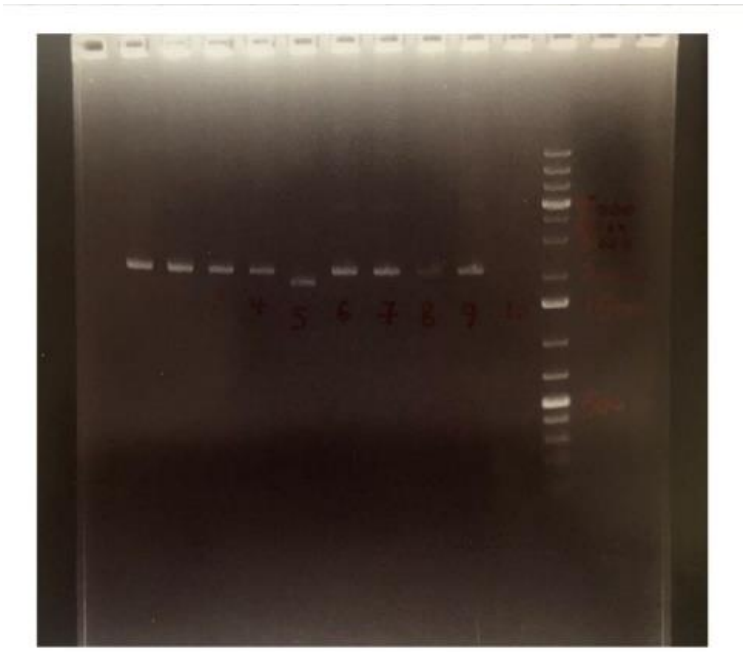

Figure 5.3 Gel electrophoresis of pGEM-T DNA digested with SalI. T_0 plants containing KO construct positive DNA were used for PCR amplification of the *OsMADS27* gRNA target region, ligated into the cloning vector pGEM-T, and after digestion with the SalI restriction enzyme to confirm the presence of inserts. The sample in lane 5 showing a smaller band is an example of an empty plasmid. All other samples are plasmids with PCR inserts.

Sequence results indicated that transgenic lines T1_3, T1_6, T1_23, T1_10_1, T1_10_2, T1_14_1, T1_21_2, T1_16_2, T1_26_1, T1_26_2, T1_16_1 had nucleotide deletions in *OsMSDS27* listed in Table 5.5 and were classified as knockout lines. T1_6 lines having a 1-bp deletion in *OsMADS27* were used for RNAseq analysis.

Alignments between WT and KO mutants are shown in Figure 4. Note that all the mentioned lines were aligned against WT sequence but only T1_3, T1_6 and T_10_1 are showed here, because all the lines with the 11 bp deletion had the same type of mutation (Figure 5.4A). Protein alignments up to pre-mature stop codons for all the KO mutants are shown in Figure 5.4B.

Table 5.5 Knockout (KO) plant samples with specific sequence deletions in *OsMADS27*.

KO samples	bp deletions
T1_6 T1_21_2	1
T1_23, T1_10_1 T1_10_2 T1_14_1 T1_16_2 T1_26_1 T1_26_2	11
T1_3	4
T1_16_1	3

Figure 5.4 Alignments of OsMADS27 WT and CRISPR/Cas9-generated knockout (KO) mutants. **A.** Red colored nucleotides are missing in KO mutants. KO_3 (T1_3) has a 4-bp deletion, KO_6 (T1_6) has 1-bp deletion, KO_10 (T1_10_1) has a 11-bp deletion and KO_16 (T1_16_1) has a 3-bp deletion. **B.** Protein alignments for KO_3, KO_6, and KO_10 mutants. Green colored amino acids in KO mutants are identical to WT amino acids. In KO_3 and KO_10, every amino acid is the same as WT until amino acid number 67. Pre-mature stop codons (*) occurred after amino acid number 94 for KO_3 and after amino acid number 69 for KO_10 mutants compared to WT. In KO_6, every amino acid is the same as WT until amino acid number 68. Pre-mature stop codon (*) occurred after amino acid number 95 for KO_6 line.

5.3.2 Overexpression of *OsMADS27* affects primary root lengths in *japonica* rice plants for generations

T_1 plants, progenies of T_0 plants, were genotyped (using the mentioned primers related to transgenic and WT plants) revealing both *OsMADS27* overexpressing (OE) and segregating wild type (WT) progenies. To include other WT controls, Kasalath seeds obtained from USDA and Krasnodarskij 3352 grown in roof-top paddies at Marquette University in Milwaukee, Wisconsin, were used. Shoot and root growth measurements were done with 14-day-old seedlings. There was no difference in shoot growth of OE, WT and segregating WT plants for both Kasalath and Krasnodarskij 3352 plants (Figure 5A & B). However, Krasnodarskij 3352 WT plants had significantly longer roots than *OsMADS27* OE plants (p = 0.02; Figure 5.5C) and segregating Kasalath WT plants had significantly longer roots than *OsMADS27* OE plants as well (p = 0.04; Figure 5.5D). These results were like those published by Chen et al. (2018) for the *japonica* subspecies where the authors concluded that overexpressing *OsMADS27* inhibits primary root growth while promoting lateral root growth. Here we show that overexpression of *OsMADS27* generates a similar root phenotype in *indica* (not previously shown) as in *japonica*.

Siblings of T_3 plants were grown until maturity and T_4 seeds were germinated and grown. Shoots and roots measurement was done with 14-day-old seedlings. Like T_1 plants, for Krasnodarskij 3352 seedlings, there was no difference in shoot length between OE and WT plants (Figure 5.6A), while the primary root of OE seedlings was significantly shorter than of WT seedlings ($p = 8.8818e^{-16}$; Figure 5.6C). However, for Kasalath seedlings, shoots of WT seedlings were significantly longer than of OE and KO seedlings (p = 0.0010053, p = 0.0017654, respectively). Interestingly, KO seedlings looked like WT plants phenotypically and shoots of KO seedlings were significantly longer than of OE plants (p = 0.0114292; Figure 5.6B). There was no difference between primary root length of WT, OE and KO seedlings (Figure 5.6D).

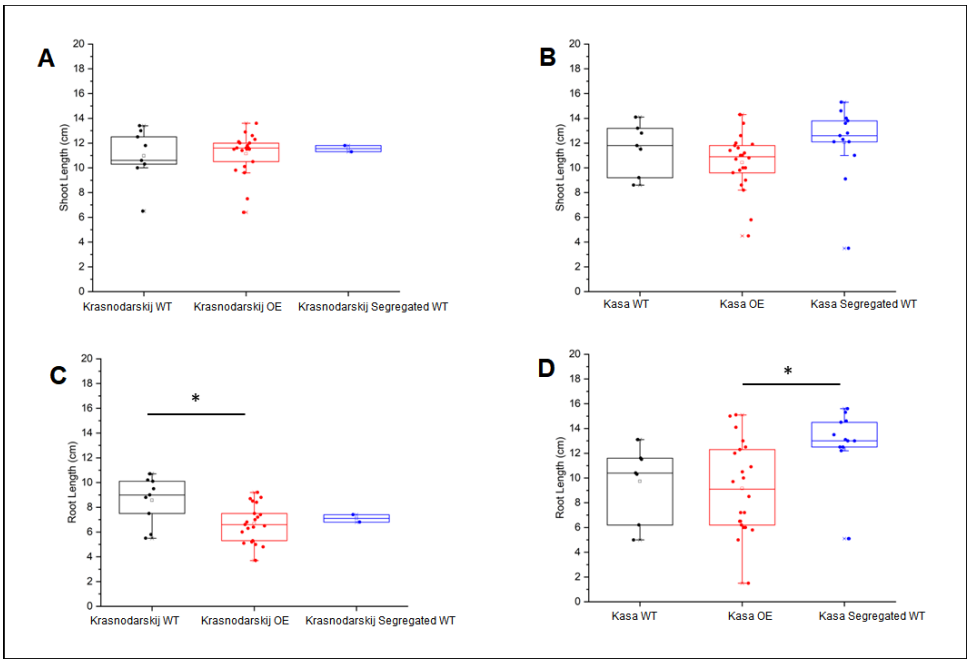

Figure 5.5 Shoot and root length phenotypes of 14-day-old *OsMADS27* overexpressing (OE) T_1 plants. Each dot is one seedling. **A & B.** Shoot lengths. No difference is seen between OE and wild type (WT) plants for both *indica* and *japonica* lines. **C & D.** Root lengths. OE plants of *japonica* and have shorter roots than WT plants. Statistical significance was determined by one-way ANOVA with post-hoc Tukey HSD tests. *, $p < 0.05$.

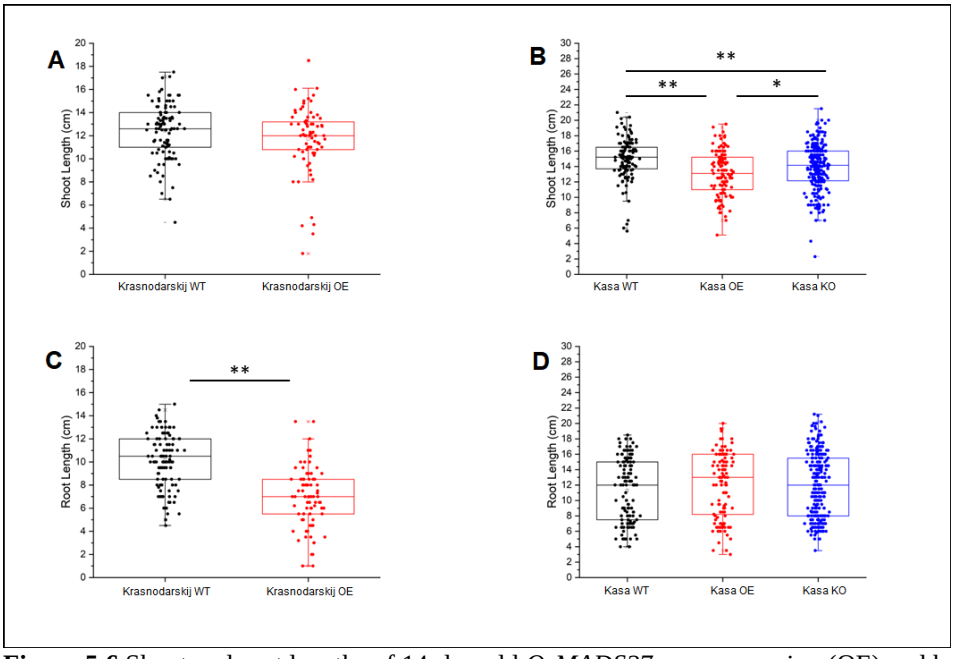

Figure 5.6 Shoot and root lengths of 14-day-old *OsMADS27* overexpressing (OE) and knockout (KO) T_4 plants. Each dot is one seedling. **A & C.** Shoot and root lengths of *japonica* OE and wild type (WT) plants. Primary root length of *japonica* OE seedlings was shorter than of WT seedlings. **B & D.** Shoot and root lengths of *indica* OE, WT and KO plants. Shoot length of *indica* OE seedlings was shorter than of WT and KO seedlings. Shoot length of *indica* KO seedlings was shorter than of WT seedlings. Statistical significance was determined by one-way ANOVA with post-hoc Tukey HSD tests. *, $p < 0.05$, **, $p < 0.01$.

5.3.3 *P. mosselii* inoculation of *OsMADS27* overexpressing rice seeds affects seedling growth of *japonica* and *indica* plants

Progenies of T_4 plants were inoculated with *P. mosselii* (named as 132) to determine the effect of this bacterium on transgenic (*OsMADS27* OE and KO) rice plants. Shoot and root length measurements were done with 14-day-old seedlings. Results showed that when *OsMADS27* OE Krasnodarskij 3352 seeds were inoculated with *P. mosselii*, shoots length was shorter compared to uninoculated OE and uninoculated and inoculated WT seedlings (Figure 5.7A). A similar pattern for primary root length was observed: inoculated OE Krasnodarskij 3352 seedlings had shorter primary roots compared to inoculated WT seedlings (Figure 5.7C). In Kasalath, inoculated OE seedlings had shorter shoots compared to inoculated and uninoculated WT seedlings (Figure 5.7B). The primary root length of OE Kasalath seedlings was shorter than of uninoculated OE seedlings (Figure 5.7D). Taken together, the results suggest that there was a negative interaction in plant growth between *P. mosselii* and *OsMADS27* OE transgenic lines.

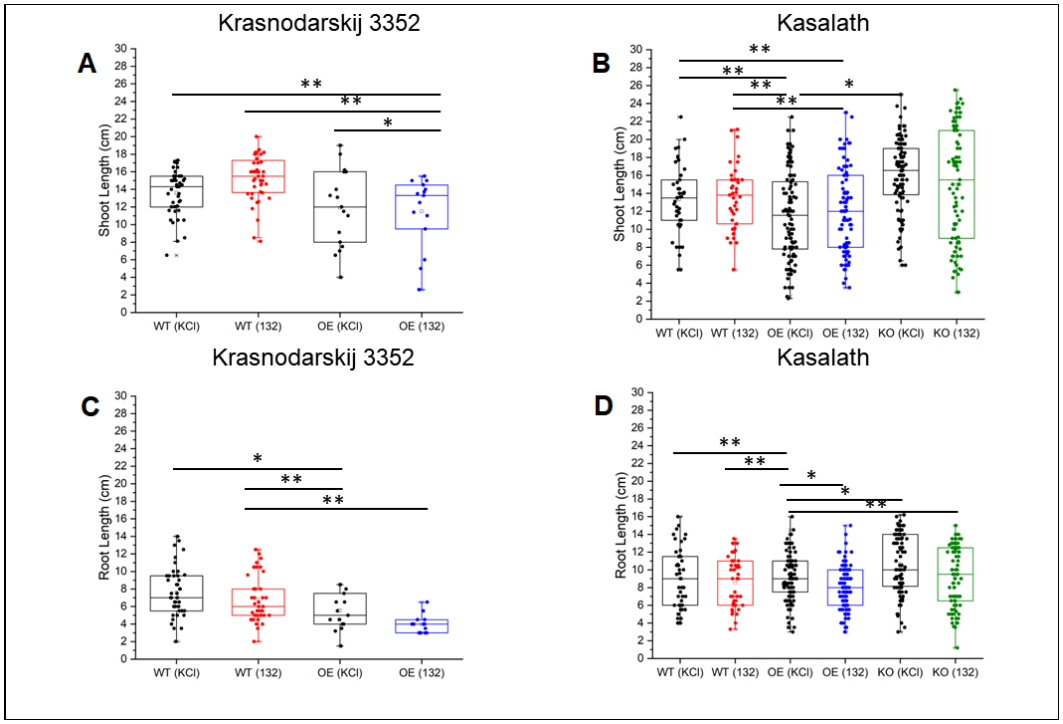

Figure 5.7 Shoot and root lengths of *P. mosselii* inoculated and uninoculated 14-day-old *OsMADS27* overexpressing (OE) and knockout (KO) T_5 plants. Each dot is one seedling. **A & C.** Shoot and root length of *P. mosselii* (132) inoculated and uninoculated control (KCl) *japonica* OE and wild type (WT) plants. *P. mosselii* affects shorter root and shoot lengths in OE *japonica* seedlings. **B & D.** Shoot and root lengths of with *P. mosselii* (132) inoculated and uninoculated control (KCl) *indica* OE, WT and KO plants. *P. mosselii* affects shorter root and shoot lengths in OE *indica* seedlings. Statistical significance was determined by one-way ANOVA with post-hoc Tukey HSD tests. *, $p < 0.05$, **, $p < 0.01$.

5.3.4 Overexpression of *OsMADS27* improved survivability of the first generation *indica* and *japonica* plants (T_1 seedlings)

T_1 seedlings of *OsMADS27* OE lines were cold stressed and evaluated for survivability after one week of recovery. WT Kasalath, segregated WT Kasalath, and OE Kasalath had 42.9%, 58.3% and 84.0% low-temperature seedling survivability (LTSS), respectively (Figure 5.8A). Krasnodarskij 3352 WT, segregated Krasnodarskij 3352 WT, and Krasnodarskij 3352 OE had 55.6%, 50.0%, and 86.4% LTSS, respectively (Figure 5.8B). These LTSS results show that overexpression of *OsMADS27* improved the cold tolerance potential of *O. sativa* seedlings. However, when cold stress was applied on T_4 seedlings, electrolyte leakage (EL) measurements showed more cell membrane damage in Krasnodarskij 3352 OE than in WT seedlings (Figure 5.9A). For Kasalath seedlings, EL measurements revealed no differences between OE and WT or KO and WT seedlings (Figure 5.9B). In this experiment, LTSS was 100% for both WT and transgenic plants. Results of salinity stress tolerance are summarized in Table 5.6. Salinity survival rates of OE and WT plants of both subspecies were the same, while Kasalath KO lines had lower survivability compared to WT seedlings.

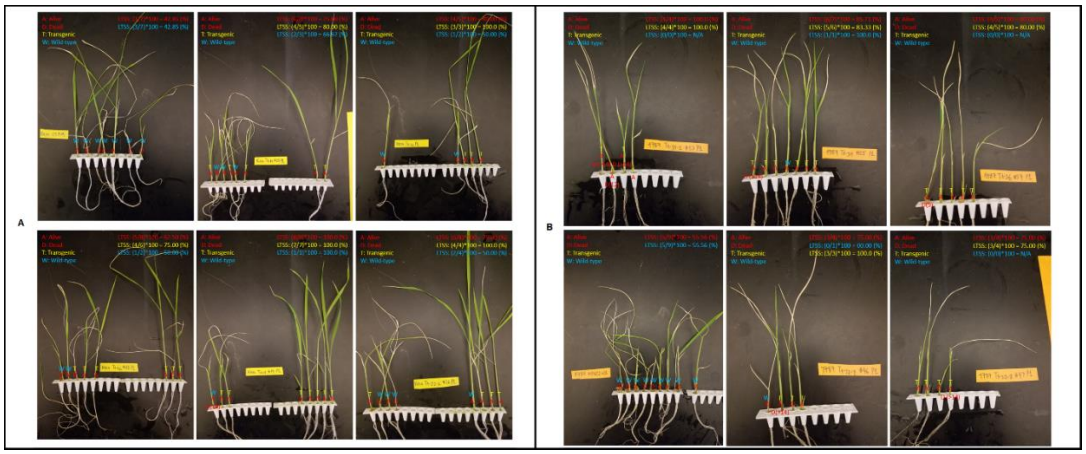

Figure 5.8 Low-temperature seedling survivability (LTSS) of *OsMADS27* overexpressing (OE) T_1 seedlings. **A.** Wild type (WT), segregated WT, and OE Kasalath lines have 42.9%, 58.3% and 84.0%, LTSS, respectively. **B.** WT, segregated WT, and OE Krasnodarskij 3352 lines have 55.6%, 50.0% and 86.4% LTSS, respectively.

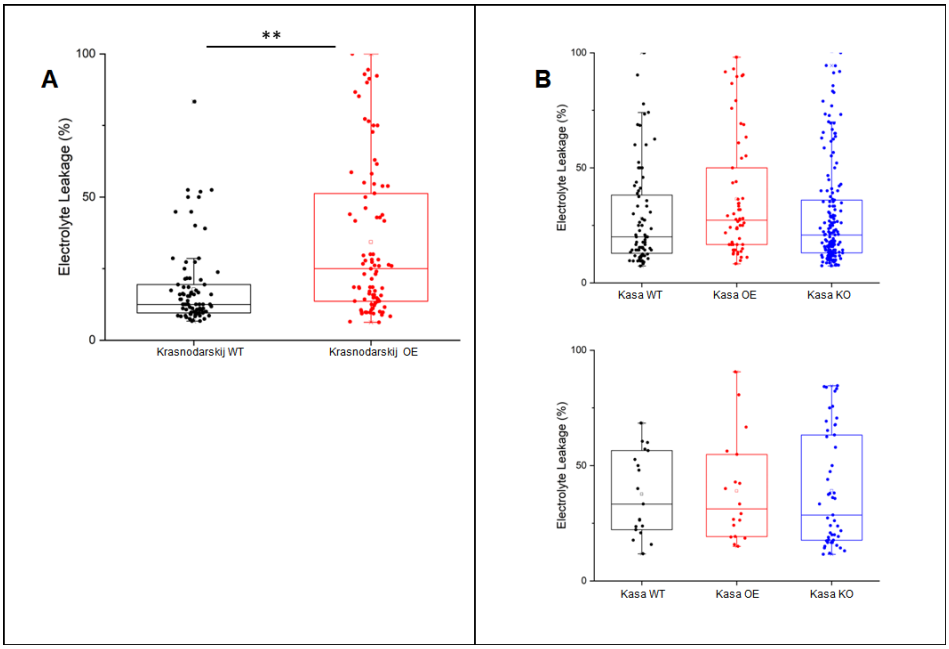

Figure 5.9 Electrolyte leakage (EL) measurements of *OsMADS27* overexpressing (OE) T_4 and knockout (KO) seedlings. **A.** EL values of Krasnodarskij 3352 OE seedlings were significantly higher than of wild type (WT) plants, indicating that the cell membrane of OE plants was more damaged than of WT plants. Seedlings were cold stressed 4 days at 4˚C (4d4˚C). **B.** EL values of WT, OE and KO Kasalath seedlings. Seedlings in the upper panel were cold stressed 2d10˚C and in the lower panel 4d10˚C. No differences are seen. Statistical significance was determined by one-way ANOVA with post-hoc Tukey HSD tests. *, $p < 0.05$, **, $p < 0.01$.

Table 5.6 Salinity tolerance *OsMADS27* overexpressing (OE) T_4, knockout (KO), and wild type (WT) seedlings (one trial)

Rice subspecies	Salinity survival % (150 mM NaCl)
Kasalath WT	62
Kasalath OE	43
Kasalath KO	28
Krasnodarskij 3352 WT	33
Krasnodarskij 3352 OE	37

5.3.5 *P. mosselii* increased cold stress survivability of *indica* wild type and *OsMADS27* overexpressing *japonica* seedlings

There was no difference in cold survival of transgenic Kasalath plants inoculated with *P. mosselii* compared to uninoculated transgenic Kasalath plants. Interestingly, inoculated Kasalath KO plants had reduced survivability compared to uninoculated control plants. As previously shown (chapter 3; Maghboli Balasjin et al. 2023, in revision), cold tolerance was improved in inoculated Kasalath WT plants compared to uninoculated WT Kasalath plants. Inoculated and uninoculated Krasnodarskij 3352 WT plants had

similar survival scores. Interestingly, LTSS of inoculated Krasnodarskij 3352 OE plants was significantly (p = 0.04) higher than of uninoculated Krasnodarskij 3352 OE plants (Table 5.7).

Table 5.7 % Low-temperature seedling survivability (LTSS) averages of *OsMADS27* overexpressing (OE) T_5, knockout (KO), and wild type plants. Cold stress was 2d10˚C and 4d4˚C for *indica* and *japonica,* respectively. LTSS of inoculated Krasnodarskij 3352 OE was significantly higher than uninoculated counterparts (2 trials). Statistical significance was determined by one-way ANOVA with post-hoc Tukey HSD tests. *, $p < 0.05$, **, $p < 0.01$.

Rice subspecies	Inoculated with *P. mosselii*	Uninoculated (KCl)
Kasalath WT	49.5	12.5
Kasalath OE	7	8.5
Kasalath KO	37	59
Krasnodarskij 3352 WT	96.5	93.5
Krasnodarskij 3352 OE	100	58.5

Salinity tolerance of inoculated Krasnodarskij 3352 OE and WT plants was not different from uninoculated Krasnodarskij 3352 OE and WT plants. In this experiment, none of the Kasalath plants survived (Table 5.8).

Table 5.8 Salinity survival (%) average of T5 transgenic *OsMADS27* seedlings. *Japonica* and *indica* plants were stressed in 150 mM and 155 mM NaCl, respectively (2 trials).

Rice subspecies	Inoculated with *P. mosselii*	Uninoculated (KCl)
Kasalath WT	0	0
Kasalath OE	0	0
Kasalath KO	0	0
Krasnodarskij 3352 WT	33	41.09
Krasnodarskij 3352 OE	20	40

Taken together, *P. mosselii* improved cold tolerance of Krasnodarskij 3352 OE plants but had no effect on either Kasalath KO or Kasalath OE plants.

5.3.6 RNAseq results confirmed *OsMADS27* OE phenotype and revealed potential *OsMADS27* target genes in Kasalath

To understand the *OsMADS27* mechanism of action inside *O. sativa*, global RNA sequencing (RNAseq) analyses using T_3 transgenic and WT control seedlings were done. RNAseq results indicated that under warm conditions, mRNA levels of *OsMADS27* were 5.72-times higher in Kasalath OE than WT plants (log2FoldChange= 2.5224; p_{adj}= 1.27xE^{-12}), indication that the MAC promoter driving the *OsMADS27*-cDNA was functional in Kasalath and led to significant upregulation. RNAseq data moreover showed that the OE lines contained pRGEB31 sequences, indicating the plants were indeed transgenic. After a 2d10˚C cold stress period, mRNA levels of *OsMADS27* were 10.17-times higher in Kasalath OE

than WT plants (log2FoldChange= 3.3405; p_{adj}= 2.05xE^{-04}), possibly indicating the MAC promoter was cold-responsive. This was further confirmed when cold induction of *OsMADS27* was compared between WT and OE lines: *OsMADS27* mRNA levels were 6.48-fold higher in WT plants (log2FoldChange= 2.6944; p_{adj}= 3.82xE^{-05}) but 8.75-fold higher in OE plants (log2FoldChange= 3.1200; p_{adj}= 1.33xE^{-17}) after a 2d10˚C treatment. In agreement with our sequencing analysis revealing a 1-bp deletion in the coding region of *OsMADS27* in the KO line used for phenotypic analyses, *OsMASDS27* mRNA levels were lower in the KO line than in WT plants under both warm and transient cold growth conditions, indicating that the resulting premature stop codon might have led to a partial non-sense mediated mRNA decay. On the other hand, the Krasnodarskij 3352 OE line used for RNAseq analysis did not have a higher *OsMADS27* mRNA abundance than WT plants nor did it have pRGEB31 associated sequences. Because the seedling growth phenotype of the OE line used for phenotypic analyses was similar to that previously published by Chen et al. (2018) for another *japonica* line, it is possible that RNA from a segregated WT instead of an OE plant was sent out for RNAseq analysis.

The global RNAseq analysis allowed us to identify differentially expressed genes (DEGs) that were either up- or down-regulated under warm control growth conditions in *OsMADS27* OE Kasalath compared to WT plants. A Gene Ontology (GO) enrichment analysis showed that for “biological process”, defense genes were significantly upregulated, indicating that those might be direct or indirect targets of the *OsMASDS27* transcription factor (Figure 5.10). Conversely, photosynthesis related genes were downregulated in *OsMADS27* OE lines (Figure 5.11). Moreover, reactive oxygen species (ROS) detoxifying peroxidases, specifically, *OsPRX25* (Os02g023700), *OsPRX91* (Os06g0695400), and *OsPRX92* (Os06g0695300*)*, were upregulated in *OsMADS27* OE lines compared to WT plants. Taken together, overexpression of the MADS box transcription factor encoding gene *OsMADS27* activated a general biotic and abiotic stress response system in transgenic plants.

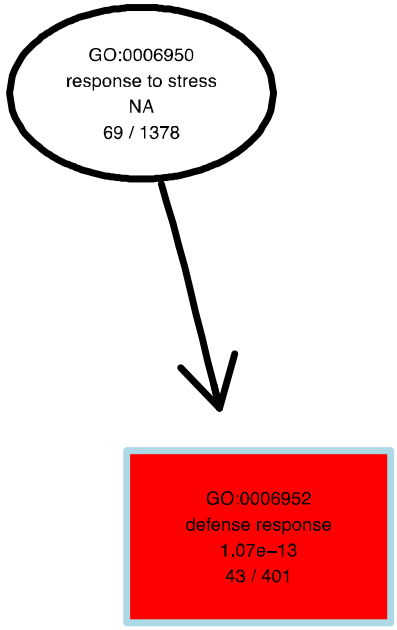

Figure 5.10 Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). Shown is a partial analysis of genes upregulated in *OsMADS27* overexpressing lines compared to wild type Kasalath plants, indicating that a significant number of defense genes were upregulated under standard warm temperature growth conditions (28˚C/25˚C 12h/12h light/dark cycles).

Figure 5.11 Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). Shown is a partial analysis of genes downregulated in *OsMADS27* overexpressing lines compared to wild type Kasalath plants, indicating that a significant number of photosynthesis related genes were downregulated under standard warm temperature growth conditions (28˚C/25˚C 12h/12h light/dark cycles).

5.4 Discussion

MADS transcription factors (TFs) play important roles in different biological processes in animals, fungi and plants. Due to evolutionary reasons, MADS TFs have been more developed in plants (Lai et al., 2019). There are two main lineages of these MADS TFs known as type I and type II (Masiero et al., 2011). Type II genes include MIKC-type genes in plants, which play an important role in flowering time and floral development (Masiero et al., 2011; H. Shan et al., 2009). Type II genes are longer and contain five to eight exons compared to type I genes which are shorter. In addition, type II genes in plants have been conserved containing four domains (M,I,K,C) with different functions (Masiero et al., 2011).

It is known that MADS TFs are involved in plant reproduction and stress responses (Tang et al., 2019). MADS TFs are mostly expressed in vegetative tissues (Yu et al., 2014). Studies by Gan et al. (2012) and Yu et al. (2014) showed that overexpression of the *ANR1* MADS-Box gene in *Arabidopsis* specifically increased lateral root (LR) growth when nitrate (NO_3 -) is supplemented in the soil. *OsMADS27* is one of the TFs in *Oryza sativa*, which is similar to *ANR1* in *Arabidopsis* (Yu et al., 2014). It is known that *OsMADS27* is responsive to NO_3 - and when *O. sativa* plants (ssp. *japonica* cv. Nipponbare) are supplied with NO_3, the primary root (PR) growth is inhibited while the LR growth is increased through abscisic acid (ABA) hormone signaling (Yu et al. 2014; Chen et al. 2018). It is also known that overexpression of *OsMADS27* increases salt tolerance in the *japonica* variety Nipponbare (Chen et al. 2018). Therefore, this TF helps rice plants to alleviate abiotic stresses.

According to the Genome-wide association studies (GWAS) analysis by Shimoyama et al. (2020), a single nucleotide polymorphism (SNP) within *OsMADS27* was strongly correlated with varying cold stress tolerance potentials. Also, preliminary expression analyses described in the Marquette University PhD dissertation of Yao Shi (2020) showed that transcript levels of *OsMADS27* increased 1.6-fold and 2.3-fold in Zhonghua 11 (*japonica*; 4d4˚C cold stress) and Kasalath (*indica*; 2d10˚C cold stress) seedlings, respectively. Based on these results, we decided to generate transgenic rice plants (Kasalath and Krasnodarskij 3352 representing *indica* and *japonica*, respectively) overexpressing (OE) and knocking out (KO) *OsMADS*27 to determine whether the gene had a positive effect on cold tolerance potential of rice plants.

Our results show that *OsMADS27* OE T_1 lines of both the *indica* and *japonica* subspecies showed improvement in cold temperature survivability compared to wild type plants (Figure 5.8). Interestingly both Kasalath and Krasnodarskij 3352 OE lines had shorter roots than their WT counterparts (Figure 5.5). A shorter primary root of *japonica* OE seedlings was previously reported by Chen et al. (2018), in agreement with our results.

RNAseq analyses of *OsMADS27* OE Kasalath T_3 lines and Kasalath KO lines confirmed upregulation in OE and downregulation in KO plants. An enrichment analysis of differentially expressed genes showed that unstressed *OsMADS27* OE Kasalath plants had a significant upregulation of defense genes (Figure 5.10) as well as ROS detoxifying genes (peroxidases), while photosynthesis related genes were downregulated. This suggests two things: first, abiotic and biotic stress defense as well as photosynthesis related genes are direct or indirect targets of the MADS box transcription factor *OsMADS27*; and second, activation of general stress defense pathways prepares unstressed rice plants overexpressing *OsMADS27* for a subsequent cold stress exposure, resulting in higher seedling survivability compared to wild type plants. As rice plants are mostly grown in tropical areas, low temperature as an abiotic stress cause injuries in rice plants (Sanghera et al., 2011). In these conditions, reactive oxygen species (ROS) will be overproduced and one of the important plants defense mechanisms is to scavenge excess amounts of ROS through peroxidase enzymes (Hasanuzzaman et al., 2017). Upregulation of peroxidase genes in *OsMADS27* OE Kasalath seedlings might have a "priming" effect to allow OE plants to better cope with low temperature stress. A tradeoff effect from this "priming" effect might be the growth retardation observed in *OsMADS27* OE seedlings. Shoot and root length measurements of T_4 plants showed that primary roots of Krasnodarskij 3352 OE seedlings were shorter than WT seedlings and shoots of Kasalath OE and KO seedlings were shorter than WT plants (Figure 5.6). Primary root play crucial role in water and nutrient uptake (Guo et al., 2020). According to our RNAseq results, *OsMADS27* upregulates ROS scavenging related genes under warm conditions and there is a connection between root growth regulation, auxin biosynthesis and ROS homeostasis (Guo et al., 2020).

As mentioned earlier, lateral root growth in overexpressing rice plants with *OsMADS27* is induced in response to NO_3- availability (Chen et al. 2018) and it is known that some PGPB are able to provide NO_3- to plants through nitrogen-fixation. According to our previous studies (Chapter 2 & 3, (Maghboli

Balasjin et al. 2022; Maghboli Balasjin et al. 2023, in revision) *P. mosselli* was one specific nitrogen-fixing PGPB with the ability of improving cold tolerance of both *indica* and *japonica* varietal plants. We hypothesized that there will be a positive relationship between *P. mosselii* and *OsMADS27* in rice plants. Interestingly, inoculation of rice seeds with *P. mosselii* had a positive effect on LTSS of *OsMADS27* OE T_5 Krasnodarskij seedlings compared to uninoculated Krasnodarskij OE seedlings (Table 5.7). The reason for the different behavior of *P. mosselii* in the two rice subspecies could be due to different gene interactions between *P. mosselii* and rice plants. Overexpression of *OsMADS27* might upregulate a common as well as a different set of target genes in *indica* and *japonica* lines, and these expressions differences might affect different interactions with *P. mosselii* genes.

In addition to cold stress, salinity stress was the second abiotic stressor that we applied to *OsMADS27* overexpressed lines and there was no difference in salt tolerance survivability rate between OE and WT lines, even in inoculated transgenic plants. There could be different reasons for not responding to salt stress. One of the reasons could be salt-ion toxicity for the two rice varieties analyzed at NaCl concentrations of 150 and 155 mM. In general, there are two phases for salt tolerance mechanisms in plants. Phase I is the accumulation of salts in plant's older leaves (osmotic stress) and phase II is the absorption of saline water by younger leaves which leads to salt-ion toxicity (de Costa et al., 2012). Accumulation of Na^+ ions in plant tissues inhibits photosynthesis and increases ROS levels in plant tissues (Numan et al., 2018). Overexpression of *OsMADS27* in Kasalath and Krasnodarskij 3352 rice varieties may lead to salt-ion toxicity when plants are exposed to excess amounts of NaCl and therefore photosynthesis is inhibited. The other reason could be the concentration of NaCl (150 – 155 mM) which might be toxic for these two rice varieties. Also, the length of salt stress may affect these two *OsMADS27* transgenic varieties negatively.

The results of this study are preliminary and further research and experiments (phenotypic and molecular characterization) need to be done to understand the mechanisms of action of *OsMADS27* in cold temperature, as well as interactions between *P. mosselii* genes and *OsMADS27*.

Chapter 6: Discussion

Overview

In this study, two different strategies for cold tolerance in rice plants were investigated. The first strategy was to apply plant growth promoting bacteria (PGPB) isolated from two rice subspecies varieties to normal growth and cold stressed plants. The second strategy was gene manipulation, in which one cold related transcription factor was selected and overexpressed or knocked out in rice plants to determine its effect on cold tolerance. To be able to grow rice and similar crop plants under cold temperatures and to avoid damages to the ecosystem, the use of PGPB and creating transgenic plants, which influence plant defense, stress tolerance and grain yield positively, will be beneficial and an environment-friendly approach (Jaganathan et al. 2018; Ngalimat et al. 2021).

Previously identified rice-associated PGPB genera include *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Pantoea*, *Acinetobacter*, *Mitsuaria*, *Azospirillum*, *Azoarcus*, *Zoogloea*, *Enterobacter*, *Micrococcus*, *Rahnella*, *Rhizobium*, *Exiguobacterium*, *Chryseobacterium*, *Ralstonia*, *Kocuria*, *Serratia*, *Burkholderia*, *Chryseobacterium*, *Streptomyces*, and *Ochrobactrum*, all of which improve rice growth and stress tolerance (Awlachew & Mengistie, 2022; de Souza, Meyer, et al., 2015; Hernández-Forte et al., 2022; Hussain et al., 2022; Y. Jha, 2012; Malik et al., 1997; Ngalimat et al., 2021; Ouyabe et al., 2020).

In the past few decades, the use of genetic tools and techniques to manipulate genes in plants such as *Agrobacterium*-mediated transformation and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) (CRISPR/Cas) methods to create transgenic plants made it easier for scientists to understand the role of genes and their mechanisms of action for improvement of crop species (Duan et al. 2012; Jaganathan et al. 2018; Romero and Gatica-Arias 2019).

This work has identified the specificity of rice-bacteria interactions on normal growth of rice (Chapter 2) and possible mechanisms of one specific PGPB, *Pseudomonas mosselii*, which was initially isolated from leaves of the cold treated *indica* type (*aus*) Carolino 164 rice variety (Chapter 3 & 4). In addition, I made phenotypical and molecular characterizations of transgenic rice plants with one specific transcription factor (TF), *OsMADS27*, which was previously identified as a cold-temperature induced TF, by either overexpressing it or knocking it out in rice plants (Chapter 5).

6.1 Plant growth promoting bacterial functions inside rice plants are rice genotype dependent

It is known that there are genes in each organism which regulate the interactions with other organisms (Cregger et al., 2021). Plants are hosts for different microorganisms. A small portion of these microorganisms get benefits for their own survival, while others have symbiotic relationship with plants also known as plant growth promoting microbes (M. Sharma et al., 2020). Plants produce several organic and inorganic compounds and create a suitable environment for microbial colonization (M. Sharma et al., 2020). Some of these microbes (mostly bacteria) are known as endophytes, which inhabit the inside of plant tissues as it is a non-competitive environment for them, due to reduced diversity of microorganisms in the endosphere compared to the rhizosphere (M. Sharma et al., 2020). These bacterial endophytes, are able to benefit plants through nitrogen fixation, solubilizing inorganic phosphate, production of siderophores for iron uptake and enhancing growth of the plants by producing plant hormones (M. Sharma et al., 2020). Endophytic bacteria are specific to plants, because plants select them by producing exudates that attract them and get benefits from them under stress conditions (Orozco-Mosqueda & Santoyo, 2021). Some endophytes are seed-borne which means plants inherit microbes through seed to seedling vertical transmission (Dumigan & Deyholos, 2022).

According to studies by Orozco-Mosqueda and Santoyo (2021), up until now 5 bacterial endophytes including *Azoarcus* sp. BH72, *Azospirillum* sp. B510, *Burkholderia* spp. KJ006, *Pantoea ananatis* AMG521 and *Pseudomonas stutzeri* A1501 that were isolated from *O. sativa*, can benefit rice plants through nitrogen fixation, phytohormone secretion, ACC deaminase, IAA and siderophore production. None of these studies have investigated the influence of local rice endophytic bacteria in growth and development on their own host and other related rice varietal plants.

One hypothesis of this study (Chapter 2) was that PGPB that were isolated from one specific rice variety, do not necessarily benefit their own host. To test the hypothesis, 140 bacteria associated with root surface and inner parts of roots and leaves were isolated from *japonica* (Krasnodarskij 3352) and *indica* (Carolino 164), which are the two members of *O. sativa* subspecies. 25 of these bacteria were characterized as phosphate solubilizing bacteria and only 5 with the most promising plant growth abilities were identified through whole genome sequencing. These 5 PGPB (*Pseudomonas mosselii*, *Paenibacillus rigui*, *Brevibacilllus* sp., *Microvirga* sp., *Paenibacillus graminis*) were inoculated to either Carolino 164 or

Krasnodarskij 3352 as well as other related rice varietal plants including *japonica* (Zhonghua 11) and *indica* (Kasalath). Results showed that *Paenibacillus rigui* and *Microvirga* sp. improved shoot growth of Kasalath plants compared to their uninoculated Kasalath counterparts. *Pseudomonas mosselii* increased shoot growth of Krasnodarskij 3352 plants. Interestingly, *Pseudomonas mosselii* and *Paenibacillus rigui* caused dwarf roots in Kasalath and Zhonghua 11 varietal plants, while *Microvirga* sp. and *Paenibacillus graminis* improved primary root growth in Krasnodarskij 3352 plants, while decreased biomass (dry weight root/shoot ratio) in Carolino 164 plants. *Pseudomonas mosselii* increased biomass of Zhonghua 11 varietal plants.

All 5 PGPB showed different behaviors (not necessarily positive effects) when they were inoculated to either their own host or other related hosts during plant growth and development. As a conclusion, not all local plant growth promoting endophytes always benefit their own host and depending on the host and bacterial genotype(s), they can either benefit (such as improving growth and development) or harm (such as root or shoot growth inhibition) the plant.

6.2 Mechanisms of *Pseudomonas mosselii* in improving rice cold stress tolerance

There are studies that have shown the positive influence of *Pseudomonas mosselii* on growth and biotic and abiotic stress tolerance of different crops (Alaa, 2018; B. K. Jha et al., 2009; Karkera et al., 2013; Kumar & Meena, 2019; Lal et al., 2016; S. Li et al., 2023; W. Li et al., 2020; Torre-ruiz et al., 2016; Wei et al., 2018). To the best of our knowledge, none of these studies have assessed potential mechanisms of *P. mosselii* as the local *indica* Carolino 164 endophyte on growth and cold tolerance of its own and other *O. sativa* varietal hosts.

The hypothesis for this study (Chapters 3 & 4) was that specific psychrotolerant rice growth promoting endophyte *(Pseudomonas mosselii)* contributes to the success of cold tolerance of different rice varieties through different mechanisms. According to the results, four potential mechanisms have been identified for *P. mosselii* inoculated into two different *O. sativa* varietal subspecies for aiding cold tolerance. According to the RASTtk (Brettin et al., 2015) genome analysis of *P. mosselii*, this bacterium contains genes related to “proline synthesis”, “Proline, 4-hydroxyproline uptake and utilization”, “glutathione synthesis”, which act as defense molecules against excess amounts of reactive oxygen species (ROS), and genes for “high affinity phosphate transporter and control of PHO regulon”, which is directly

induced for the uptake of inorganic phosphate. In addition to the mentioned mechanisms, according to antiSMASH analysis (Blin et al., 2021), *P. mosselii* produces secondary metabolites with antifungal properties such as “pseudopyronine”, “pseudomonine”, “tolaasin” and “fragin”.

My results (Chapter 3) showed that when *P. mosselii* was inoculated into *indica* (Carolino 164) and *japonica* (Krasnodarskij 3352) varietal plants, the rate of survivability increased to 66% and 74%, respectively, compared to their uninoculated control plants (55% and 34% for uninoculated *indica* and *japonica* plants, respectively). Also, the percentage of electrolyte leakage (EL) decreased to 14% for inoculated *indica* (compared to 18% for uninoculated *indica*) and 16% for inoculated *japonica* plants (compared to 20% for uninoculated *japonica*). From metabolic assays, the level of antioxidants such as proline and reduced glutathione increased in *P. mosselii* inoculated *japonica* and *indica* plants, respectively, before cold stress. Interestingly, these results were compatible with the percentage of ROS levels in inoculated plants during the recovery period. For both inoculated *indica* and *japonica* plants, the amount of ROS level was significantly higher ($p < 0.01$) before cold but significantly lower after cold and the recovery period compared to their control plants, indicating that uninoculated control plants were more under stress and injured more than plants inoculated by *P. mosselii*. Moreover, when *japonica* plants inoculated with *P. mosselii* grew in modified Murashige and Skoog medium containing insoluble $Ca_3(PO_4)_2$ (calcium phosphate), they had more soluble phosphate in their tissues compared to uninoculated *japonica* control plants before cold stress. Taken together, proline, reduced glutathione and soluble inorganic phosphate are known to improve cold tolerance of the plants (Trivedi and Pandey 2007; Hayat et al. 2012; Hasanuzzaman et al. 2017). According to the results of this study, *P. mosselii* had a positive effect on cold tolerance of both *indica* and *japonica* plants through increasing the amounts of proline, reduced glutathione, and soluble phosphate when plants were in normal conditions, right before cold stress. This indicated a role of *P. mosselii* in “priming” the defense by the plants, which improved responses to forthcoming environmental challenges. Generally, the *indica* subspecies is cold sensitive and according to Phan & Schläppi (2021), at the end of a 7-day cold stress period, *indica* plants have lower catalase (CAT) and anthocyanin (ANT) activities as a measure of enzymatic and non-enzymatic antioxidant activities compared to plants from the *japonica* subspecies. However, at the beginning of the cold period, *indica* plants have high levels of antioxidant activities to turn over ROS while *japonica* plants have low levels of

antioxidant activities and high levels of ROS to activate downstream stress tolerance response mechanisms (Phan & Schläppi, unpublished). Therefore, compared to *japonica*, *indica* plants "panic" and want to scavenge excess amounts of ROS accumulating in their tissues as soon as possible instead of using ROS as signaling molecules. Reduced glutathione is a non-enzymatic antioxidant involved in ROS scavenging as well as formation of phytochelatins, which inhibit another source of ROS formation in plants by chelating heavy metal ions (Das & Roychoudhury, 2014). This could be one potential reason why *P. mosselii* applies different mechanisms in *indica* and *japonica* for cold tolerance improvement, which can be connected to genotype difference between the two subspecies of rice.

Furthermore, my results (Chapter 4) showed another possible mechanism of *P. mosselii* in improving cold tolerance of *japonica* plants. Based on *in vitro* antifungal assays, at room temperature (22˚C - 23˚C), live cells of *P. mosselii* had antagonism against spore germination and vegetative growth of *Fusarium fujikuroi* [which is known to cause Bakanae disease in rice plants (Chung et al., 2016)] and spore germination of *Talaromyces aurantiacus* (which is known as phosphate solubilizer [Kaur et al., 2022)] that were isolated from *indica* Carolino 164 and *japonica* Krasnodarskij 3352 plants, respectively. It is worth mentioning that *T. aurantiacus* is known as a plant growth promoting fungus as it solubilizes phosphate in the rhizosphere of moso bamboo (Y. Zhang et al., 2018) and produces antimicrobial compounds in the rhizosphere of *Ocimum tenuiflorum* to fight against *Staphylococcus aureus* and *Escherichia coli* (N. Rani et al., 2019). However, according to the results of this study, *T. aurantiacus* can be considered a rice pathogen as it harmed control *japonica* plants compared to *P. mosselii* inoculated *japonica* plants and initial concentration of *T. aurantiacus* was not known. Interestingly, heat-treated dead cells of *P. mosselii* had antagonism against spore germination of *T. aurantiacus* at room temperature. The main goal was to investigate whether rice plants became resistant to these phytopathogens when they were inoculated with *P. mosselii* and survived better after being exposed to cold temperatures (4˚C and 10˚C). For this reason, *in vitro* antifungal assays were done at low temperatures and the same results as room temperature were obtained. Live cells of *P. mosselii* inhibited spore germination and vegetative growth of *F. fujikuroi* at low temperatures (4˚C and 10˚C) and inhibited only spore germination of *T. aurantiacus* at 10˚C.

According to *in vivo* plant assays, *P. mosselii* inoculated *japonica* plants survived better (78% and 70% survival) when treated with *T. aurantiacus* and *F. fujikuroi*, respectively, compared to untreated

control plants (52% and 55% survival) after 21 days of recovery. However, *P. mosselii* inoculated *indica* plants had a lower survival rate (44%) when treated with *F. fujikuroi*, compared to untreated control plants (70% survival) after 21 days of recovery.

Taken together, there could be three scenarios for antifungal activity of *P. mosselii*: (1) Because *P. mosselii* inoculated *japonica* plants survived better than *indica* plants when treated with the two phytopathogens, the interactions between microorganisms and rice plants are dependent on the genotype of each of these microorganisms and plants and not all PGPB behave similarly in one specific rice variety. For instance, studies of Zhang et al. (2019) revealed rice genotype-microbe specificity as *indica* plants contained different microbial communities than *japonica* plants. (2) According to results related to antagonistic influence of heat-treated dead cells of *P. mosselii* on spore germination of *T. aurantiacus*, it can be assumed that *P. mosselii* produces two or more different secondary metabolites while some are heat-resistant and have specific genotype dependent interactions with the two different pathogenic fungi. (3) Due to antagonistic effect of *P. mosselii* on vegetative growth of *F. fujikuroi*, the third scenario is that *P. mosselii* contains specific genes including N-acetylglucosamine-6-phosphate deacetylase (*NagA*), glucosamine-6-phosphate deaminase (*NagB*), predicted transcriptional regulator of N-acetylglucosamine utilization, GntR family (*NagR*) and chitin binding protein (*Chinitoluytic_Enz*), which targets the cell walls of *Fusarium fujikuroi* (chitin and N-acetylglucosamine) and therefore inhibits further hyphal growth of this fungus. In this study, two types of biotic (phytopathogens) and abiotic (cold stress) were applied to rice plants simultaneously and the influence of *P. mosselii* was evaluated. While the interactions between the PGPB and plants are genotype dependent, the reactions of beneficial and pathogenic microorganisms could harm or help a specific plant genotype. For example, it is known that different species of *Populus* contain different microbial communities, which shows that plant-microbe interactions are dependent on the host genome and, therefore, host-microbe selectivity occurs (Cregger et al., 2021).

6.3 *OsMADS27* inhibits primary root growth and increases cold tolerance of *OsMADS27* overexpressing *indica* and *japonica* lines

MADS genes play crucial roles in rice development and therefore many MADS family genes have been identified and analyzed in rice plants (Dong et al., 2023). It is known that overexpressing the *OsMADS27* transcription factor (TF) induces nitrogen uptake and controls root development through the abscisic acid (ABA) pathway and improves salt tolerance in rice plants (Alfatih et al., 2022; H. Chen et al.,

2018b). According to the PhD dissertation of Yao, 2019), the levels of *OsMADS27* transcripts increased 1.6-fold and 2.3-fold in seedlings of cold stressed *japonica* variety Zhonghua 11 and *indica* variety Kasalath, (cold stress for *japonica* was 4d4˚C, and for *indica* was 2d10˚C).

The hypothesis of this study (Chapter 5) was that synergic interactions between *Pseudomonas mosselii* and *OsMADS27* in rice plants will improve cold tolerance of rice seedlings. To test this hypothesis, *OsMADS27* overexpressing (OE) *indica* (Kasalath) and *japonica* (Krasnodarskij 3352) plants as well as *OsMADS27* knockout (KO) Kasalath plants were created to test the influence of this TF on cold tolerance of rice plants. Based on the results, cold treated T_1 *OsMADS27* OE lines of both *indica* and *japonica* survived better compared to wild type (WT) plants. In addition, primary root (PR) growth of T_1 *OsMADS27* OE lines of both *indica* and *japonica* was inhibited. Interestingly, cold treated T_3 *OsMADS27* OE Kasalath lines had more upregulated genes related to peroxidase and defense mechanism against pathogens compared to cold treated WT Kasalath plants (RNAseq analysis). Shoot height and PR growth of T_4 *OsMADS27* OE Kasalath lines were lower than of WT Kasalath plants. Furthermore, results of evaluation of influence of *P. mosselii* on *OsMADS27* OE and KO plants showed that inoculated cold treated T_5 *OsMADS27* OE Kasalath lines survived better than their uninoculated OE counterparts. According to these preliminary results, one possible mechanism of *OsMADS27* under cold stress is upregulating peroxidase genes, which are crucial for plants' defense mechanisms to scavenge the excess amount of ROS (Hasanuzzaman et al., 2017).

6.4 Overall conclusion

As mentioned above, this study was focused on two aspects of improving cold tolerance in *O. sativa* plants from subspecies: using local PGPB and gene manipulation of rice plants.

Out of 140 bacteria that were isolated from the endosphere, rhizoplane and phyllosphere of two members of *indica* and *japonica* subspecies, 25 bacteria had phosphate solubilizing activity and were further *in vitro* characterized for plant growth promoting activities. According to the results, five bacteria with the highest potential for plant growth promoting activities were selected for whole genome sequencing and for evaluating rice cold tolerance through *in vivo* plant experiments. *Pseudomonas mosselii*, isolated from *indica* leaves, significantly improved cold tolerance of *indica* and *japonica* through different mechanisms. Although the activity of *P. mosselii* was plant genotype dependent, it can be concluded that

this PGPB has the ability of "priming" the selected plants from both subspecies to respond to subsequent cold stress by increasing the amounts of two important antioxidants before cold stress exposure.

From the gene manipulation aspect, *OsMADS27* was selected to be overexpressed and knocked out in *indica* and *japonica*. Overexpression of *OsMADS27* had a positive effect on cold tolerance of both subspecies. Interestingly, this transcription factor had a positive relationship with *P. mosselii* in increasing inoculated *japonica OsMADS27*-overexpressing lines compared to uninoculated overexpressing lines. This might occur because of synergism between *OsMADS27* and *P. mosselii*: *OsMSDS27* activates defense genes in rice but requires nitrate to do so, and *P. mosselii* as a PGPB might help with nitrate uptake.

An overall summary of findings in this PhD dissertation is shown in Figure 6.1.

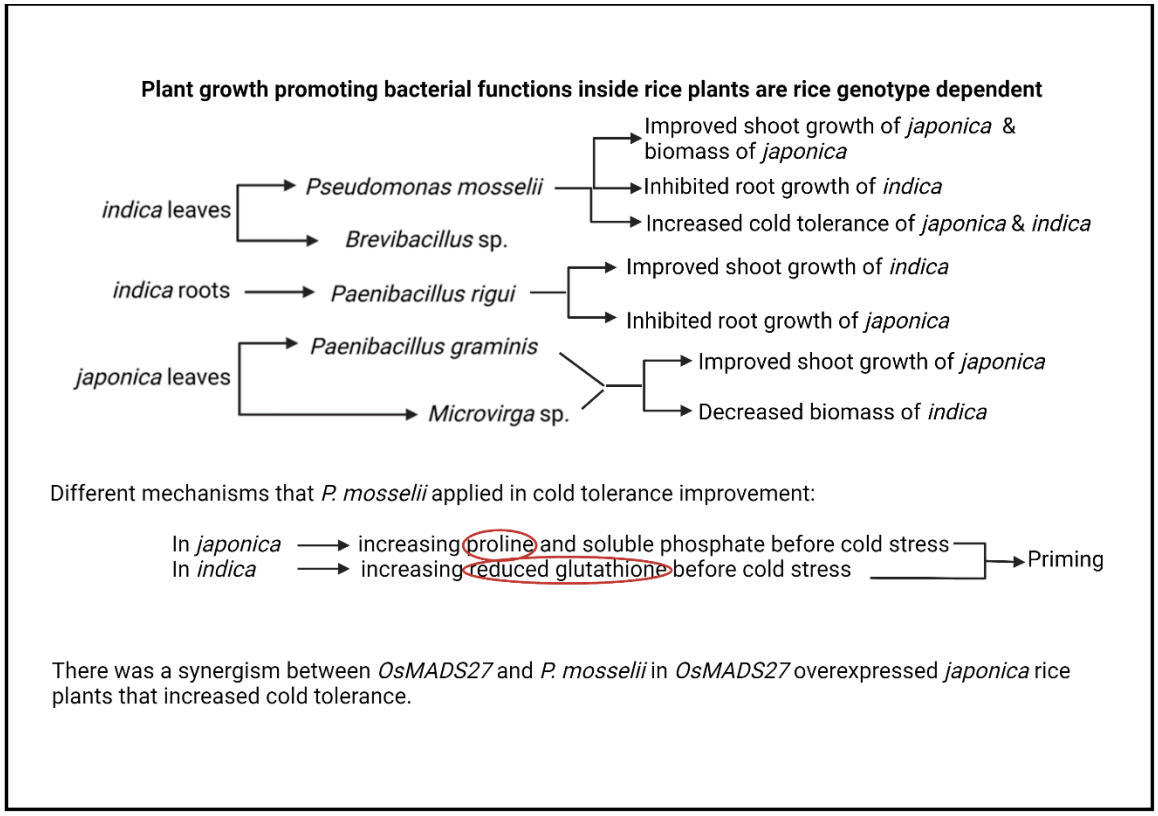

Figure 6.1 Schematic summary of findings of this study. Created with BioRender.com.

6.5 Future directions

This study was conducted on evaluating the influence of cold temperatures on young seedlings (two-week old) of two rice varieties in a controlled environment. PGPB effects and gene modifications

were also tested on young seedlings under cold stress conditions. In addition, the influence and mechanism of actions of only one single bacterium on growth and cold tolerance of rice plants was investigated. To better understand the relationships between rice plants and PGPB, further investigations need to be done to understand rice plant-microbe interactions over the long-term and during different growth stages of rice plants. It is known that rice plants contain different microbial communities at different growth stages (Aulakh et al., 2001; Delmo-Organo et al., 2017; Imchen et al., 2019), therefore, it is expected to observe different behavior and interaction with *indica* and *japonica* at their different growth stages when they are inoculated with the isolated PGPB. Also, taking the experiments to nature and rice paddies, where other factors such as temperature and humidity fluctuate and different biotic stressors and soil nutrition imbalance occur, is crucial to understand how these isolated PGPB will benefit rice plants and how dynamic plant-microbe interactions will be. The isolated PGPB showed other characteristics such as salinity tolerance, thus it is expected to help plants under different abiotic stresses. However, the synergism/antagonism of these PGPB with different varieties of soil bacteria is not known yet. Furthermore, more molecular techniques such as knocking out specific genes in *P. mosselii* related to plant cold tolerance improvement need to be done to make sure which bacterial genes are involved in cold tolerance improvement of rice plants. In this case, care must be taken to identify genes to knockout, because some genes are crucial for bacterial life cycle and if the bacterium miss those genes, its life cycle will be influenced negatively. To find mechanism(s) of defense in *P. mosselii* against phytopathogens, studies such as High-performance liquid chromatography (HPLC) and Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) need to be done as they serve as powerful analytical techniques with highly specified analysis capabilities which can identify which of the secondary metabolites of *P. mosselii* are involved in controlling *Talaromyces aurantiacus* and *Fusarium fujikuroi*. Finally, further

research and experiments (phenotypic and molecular characterization) need to be done to understand the mechanisms of action of *OsMADS27* in cold temperatures.

According to https://www.fda.gov/food/agricultural-biotechnology/how-gmos-are-regulated-united-states, multiple federal agencies work together to make sure that genetically modified (GM) plants are safe for human usage. GM developers should meet with the Food and Drug Administration (FDA) and inform them about their new GM product and then they can submit the information about their product safety to the FDA. In the last step, FDA will evaluate the submitted information and resolve potential issues with GM developers. Due to the long-term process of mass production of GM plants, applying PGPB to plants and testing interactions between different PGPB to develop organic fertilizers for plants could be the better option for improving cold stress tolerance in rice plants.

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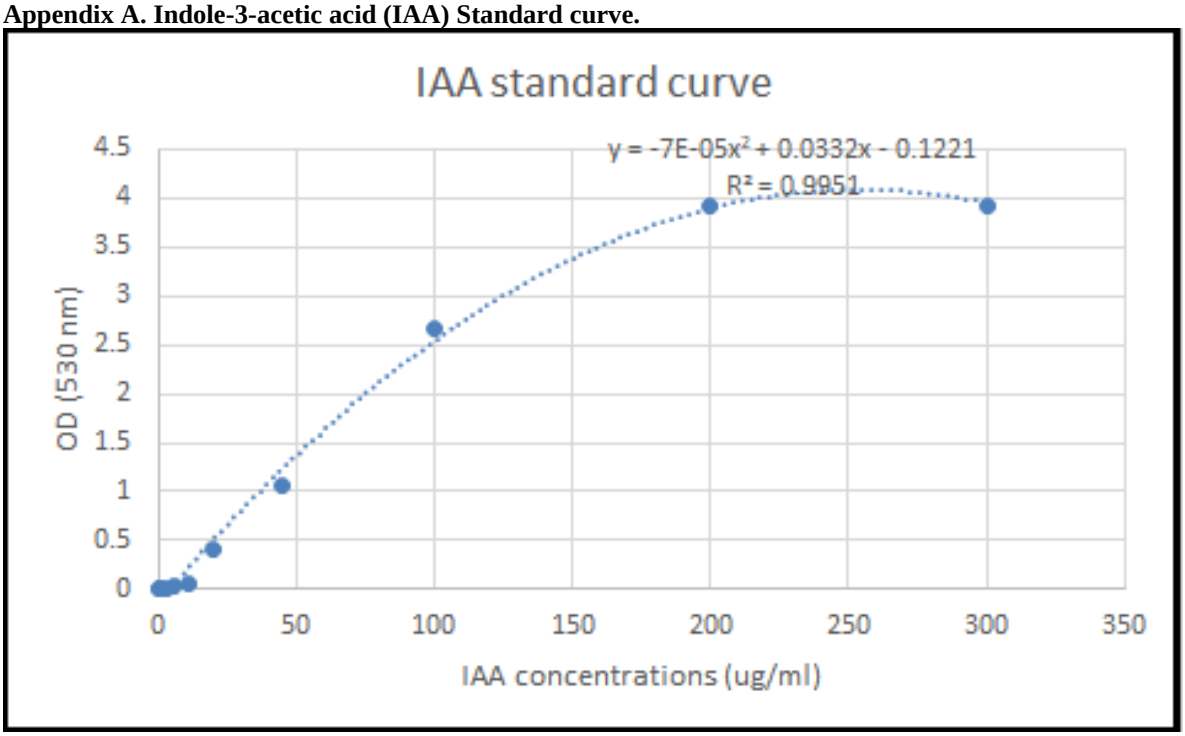
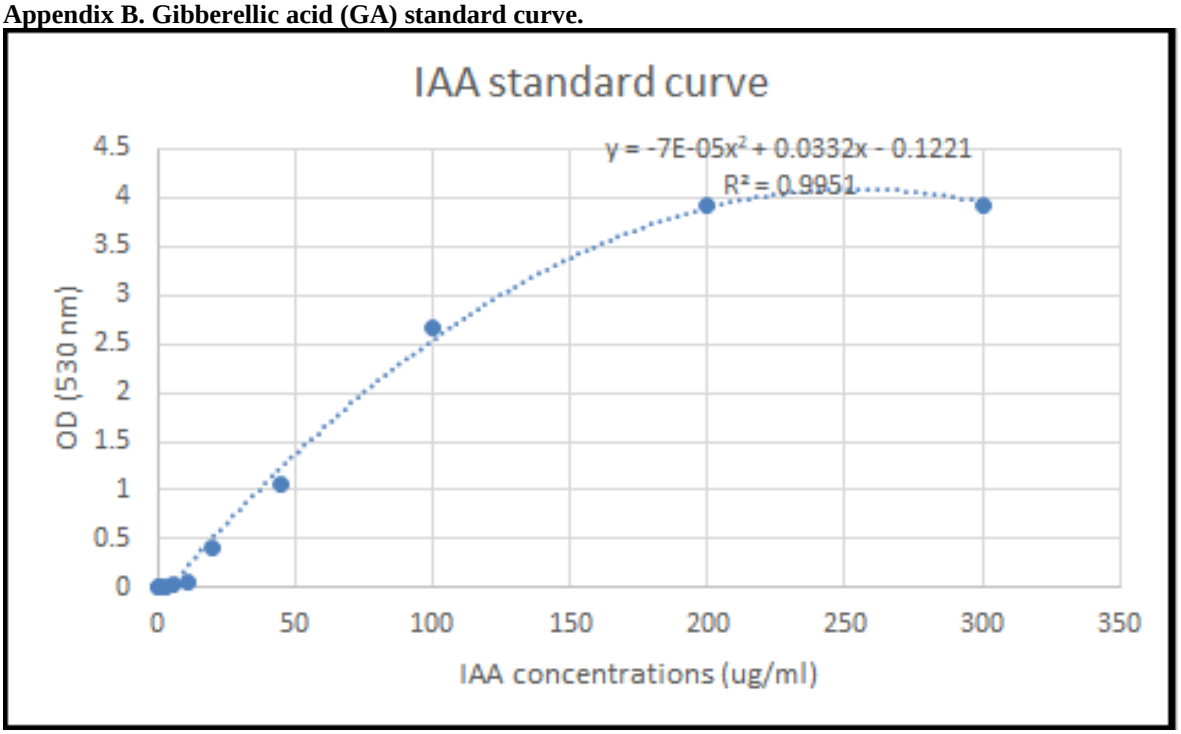
Appendix

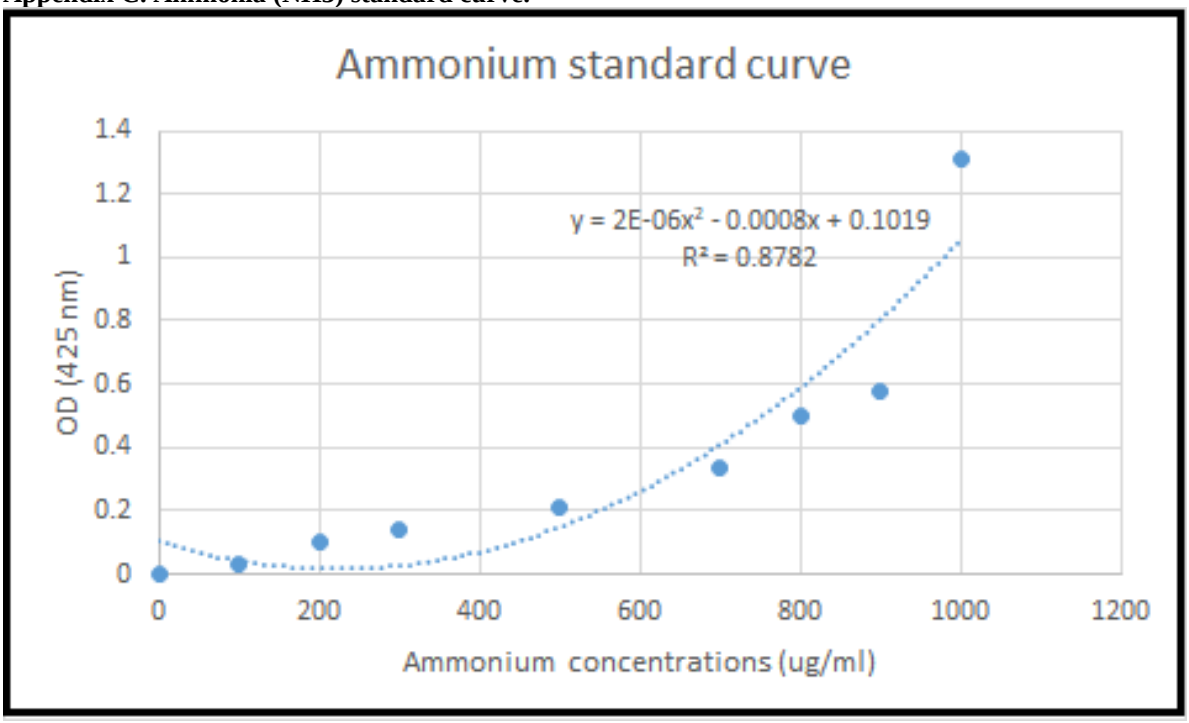
Appendix A. Indole-3-acetic acid (IAA) Standard curve.



Appendix B. Gibberellic acid (GA) standard curve.



Appendix C. Ammonia (NH3) standard curve.



Appendix D. Phenotypic characteristics of isolated bacteria from indica and japonica rhizoplane, endosphere, and phyllosphere.

***Indica* rhizoplane bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**
n00001	Circular	Slightly raised	Yellow	M	Entire	Cocci	G+
n00002	Circular	Slightly raised	Milk white	M	Entire	Cocci	G+
n00003	Irregular	Flat	Milk white	L	Undulate	Rod-shape	G-
n00004	Circular	Slightly raised	Milk white	S	Entire	Cocci	G+
n00005	Circular	Slightly raised	Milk white	S	Entire	Cocci	G+
n00006	Circular	Slightly raised	Milk white	M	Entire	Cocci	G+
n00028	Circular	Raised	Milk white	M	Entire	Cocci	G+
n00029	Circular	Raised	Yellow	S	Entire	Cocci	G+
n00030	Circular	Raised	Milk white	M	Entire	Cocci	G+
n00031	Circular	Raised	Yellow	S	Entire	Cocci	G+
n00032	Circular	Flat	Milk white	S	Entire	Cocci	G+
n00033	Circular	Raised	Milk white	M	Entire	Rod-shape	G-
n00092	Circular	Raised	Yellow	L	Entire	Cocci	G+
n00093	Circular	Raised	White	S	Entire	Cocci	G+

n00094	Circular	Raised	Milk White	M	Entire	Rod-shape	G+
n00111	Circular	Raised	Yellow	S	Entire	Cocci	G+
n00112	Circular	Umbonate	White	L	Undulate	Rod-shape	G+
n00113	Circular	Raised	Yellow	S	Entire	Cocci	G+
n00115	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00116	Circular	Raised	Yellow	S	Entire	Cocci	G+
***Indica* endosphere bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**
n00012	Circular	Flat	Milk white	S	Slightly undulate	Cocci	G-
n00013	Circular	Slightly raised	Milk white	M	Undulate	Rod-shape	G+
n00014	Circular	Raised	Milk white	S	Filiform	Rod-shape	G+
n00015	Circular	Slightly raised	Milk white	M	Undulate	Cocci	G+
n00041	Circular	Slightly Raised	Milk white	M	Lobate	Rod-shape	G+
n00042	Circular	Slightly Raised	Milk white	L	Undulate	Rod-shape	G+
n00043	Circular	Flat	Milk white	M	Undulate	Cocci	G+
n00044	Circular	Raised	White	M	Lobate	Cocci	G+
n00044-1	Circular	Raised	White	S	Entire	Rod-shape	G+
n00044-2	Circular	Raised	Milk white	M	Undulate	Diplococci	G+
n00044-3	Circular	Raised	Milk white	M	Undulate	Rod-shape	G+
n00045	Circular	Flat	Milk white	M	Undulate	Cocci	G+
n00070	Irregular	Flat	Cream	L	Filiform	Rod-shape	G+
n00106	Circular	Raised	Yellow	M	Entire	Cocci	G+
n00107	Circular	Raised	Milk White	M	Entire	Cocci	G+
n00108	Circular	Raised	Yellow	S	Entire	Cocci	G+
n00129	Circular	Flat	Transparent	S	Entire	Cocci	G+
n00130	Circular	Slightly Raised	Milk White	S	Entire	Rod-shape	G+
n00131	Circular	Slightly Raised	Milk White	S	Entire	Cocci	G+
n00147	Circular	Flat	Milk White	M	Lobate	Rod-shape	G+
n00163	Circular	Raised	White	S	Entire	Rod-shape	G+
n00173	Irregular	Slightly Raised	Milk White	S	Lobate	Rod-shape	G+
***Indica* phyllosphere bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**

n00022	Irregular	Raised	Milk white	M	Undulate	Rod-shape	G+
n00022-1	Irregular	Raised	Milk white	S	Undulate	Cocci	G+
n00023	Irregular	Raised	Milk white	M	Lobate	Rod-shape	G+
n00024	insufficient growth	-	Milk white	M	-	Cocci	G+
n00025	Irregular	Raised	Milk white	M	Undulate	Cocci	G+
n00025-1	Circular	Slightly raised	Milk white	S	Entire	Cocci	G-
n00048	Circular	Raised	Milk white	M	Lobate	Cocci	G+
n00049	Irregular	Slightly Raised	Milk white	L	Undulate	Chain Cocci	G+
n00087	Irregular	Flat	Cream	L	Filiform	Rod-shape	G+
n00109	Circular	Raised	Yellow	S	Entire	Diplo cocci	G+
n00132	Circular	Slightly Raised	Milk White	S	Undulate	Rod-shape	G-
n00157	Circular	Raised	White	M	Entire	Diplo cocci	G+
n00158	Circular	Umbonate	Milk White	M	Undulate	Rod-shape	G+
n00166	Circular	Flat	Milk White	S	Entire	Cocci	G+
n00167	Circular	Flat	White	S	Entire	Rod-shape	G+
***Japonica* rhizoplane bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**
n00007	Irregular	Umbonate	Milk white	S	Filiform	Rod-shape	G-
n00008	Irregular	Slightly raised	Milk white	M	Undulate	Cocci	G+
n00009	Irregular	Flat	Milk white	M	Undulate	Rod-shape	G-
n00010	Circular	Slightly raised	Milk white	L	Entire	Cocci	G+
n00011	Circular	Slightly raised	Milk white	M	Filiform	Rod-shape	G-
n00034	Circular	Raised	Yellow	L	Entire	Cocci	G-
n00035	Circular	Raised	Milk white	S	Entire	Cocci	G+
n00036	Circular	Raised	Yellow	M	Entire	Cocci	G+
n00039	Circular	Raised	Yellow	M	Entire	Rod-shape	G+
n00056	Circular	Raised	Milk White	S	Entire	Rod-shape	G+
n00057	Circular	Raised	Milk White	M	Entire	Rod-shape	G+
n00058	Circular	Slightly Raised	Yellow	S	Entire	Rod-shape	G+
n00059	Circular	Slightly Raised	White	S	Entire	Rod-shape	G+
n00060	Circular	Slightly Raised	Milk White	S	Entire	Cocci	G+

n00061	Circular	Slightly Raised	Milk White	S	Entire	Rod-shape	G+
n00062	Circular	Crateriform	Red	M	Entire	Rod-shape	G+
n00063	Circular	Raised	Milk White	M	Entire	Rod-shape	G-
n00064	Circular	Flat	Milk White	L	Undulate	Rod-shape	G+
n00097	Circular	Slightly Raised	Milk White	S	Entire	Rod-shape	G+
n00098	Circular	Flat	Yellow	S	Entire	Chain cocci	G+
n00099	Circular	Slightly Raised	Milk White	S	Entire	Cocci	G+
n00101	Circular	Raised	White	M	Entire	Cocci	G+
n00102	Circular	Flat	Transparent	S	Entire	Cocci	G+
n00103	Circular	Raised	Milk White	M	Entire	Rod-shape	G+
n00104	Circular	Raised	Yellow	L	Entire	Cocci	G+
n00105	Circular	Raised	White	S	Entire	Cocci	G+
n00117	Circular	Flat	Red	S	Entire	Cocci	G+
n00121	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00123	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00124	Circular	Raised	Milk White	S	Entire	Rod-shape	G+
n00125	Circular	Raised	Yellow	M	Entire	Cocci	G+
n00126	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00133	Circular	Raised	Milk White	M	Entire	Cocci	G+
n00134	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00138	Circular	Raised	Milk White	S	Entire	Rod-shape	G+
n00139	Circular	Umbonate	Milk White	M	Entire	Cocci	G+
***Japonica* endosphere bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**
n00016	Circular	Raised	Milk white	M	Entire	Rod-shape	G+
n00017	Circular	Raised	Milk white	M	Undulate	Cocci	G+
n00018	Circular	Raised	Milk white	S	Entire	Rod-shape	G-
n00019	Circular	Raised	Milk white	S	Entire	Cocci	G+
n00020	Circular	Raised	Milk white	S	Undulate	Cocci	G+
n00046	Circular	Raised	Yellow	L	Entire	Cocci	G+
n00047	Circular	Umbonate	Milk white	S	Entire	Cocci	G+

n00071	Circular	Raised	Milk White	M	Entire	Cocci	G-
n00072	Circular	Slightly Raised	Milk White	S	Entire	Cocci	G+
n00073	Circular	Flat	White	S	Entire	Cocci	G+
n00074	Circular	Umbonate	White	M	Undulate	Rod-shape	G+
n00075	Circular	Raised	Milk White	M	Undulate	Cocci	G+
n00076	Circular	Crateriform	Milk White	M	Entire	Rod-shape	G+
n00077	Circular	Flat	White	S	Entire	Rod-shape	G+
n00078	Circular	Flat	White	M	Filiform	Rod-shape	G+
n00079	Circular	Flat	Milk White	M	Entire	Rod-shape	G+
n00080	Circular	Flat	White	M	Entire	Rod-shape	G+
n00081	Circular	Flat	Yellow	S	Entire	Cocci	G+
n00082	Irregular	Flat	White	L	Undulate	Chain Cocci	G+
n00083	Irregular	Flat	Cream	L	Filiform	Rod-shape	G+
n00084	Irregular	Flat	Cream	L	Filiform	Chain Cocci	G+
n00150	Circular	Umbonate	White	L	Undulate	Chain cocci	G+
n00152	Circular	Flat	Creame	M	Undulate	Rod-shape	G+
n00153	Circular	Umbonate	White	S	Entire	Cocci	G+
n00154	Circular	Slightly raised	White	S	Entire	Cocci	G+
n00155	Circular	Flat	White	S	Undulate	Rod-shape	G+
n00156	Circular	Flat	White	S	Entire with transparent margin	Rod-shape	G+
n00164	Circular	Slightly raised	White	S	Entire	Rod-shape	G+
n00165	Circular	Raised	Milk White	S	Entire	Cocci	G+
n00174	Irregular	Slightly Raised	Milk White	S	Lobate	Rod-shape	G+
***Japonica* phyllosphere bacteria**	**Form**	**Elevation**	**Color**	**Size**	**Margin**	**Cell morphology**	**G+/G-**
n00026	Circular	Flat	Milk white	S	Entire	Cocci	G+
n00027	Circular	Slightly raised	Milk white	S	Filiform	Rod-shape	G+
n00050	Circular	Umbonate	Milk white	M	Undulate	Cocci	G-
n00051	Circular	Flat	Milk white	S	Undulate	Cocci	G+
n00052	Circular	Umbonate	Milk white	M	Entire	Rod-shape	G+
n00053	Irregular	Slightly Raised	Red	S	Undulate	Rod-shape	G+
n00054	Circular	Flat	Yellow	S	Entire	Rod-shape	G+
n00055	Circular	Flat	Milk white	S	Undulate	Rod-shape	G-

n00088	Circular	Flat	Milk White	M	Entire with transparent margins	Cocci	G+
n00089	Circular	Flat	Milk White	M	Entire with transparent margins	Chain Cocci	G+
n00162	Circular	Raised	Yellow	S	Entire	Diplo cocci	G+
n00169	Circular	Raised	White	S	Entire	Rod-shape	G+
n00170	Circular	Raised	Milk White	S	Entire	Rod-shape	G-
n00170-1	Irregular	Raised	Milk White	M	Undulate	Rod-shape	G+
n00170-2	Circular	Raised	Milk White	M	Entire	Rod-shape	G+
n00171	Circular	Raised	Yellow	S	Entire	Diplo cocci	G+
n00172	Circular	Flat	White	S	Entire	Rod-shape	G+
n00175	Circular	Raised	Yellow	S	Entire	Diplo cocci	G+